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POTASSIUM AND PHOSPHATE NUTRITION OF PINEAPPLE IN RELATION TO NITRATE AND CARBOHYDRATE RESERVES

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(WITH TWENTY-FIVE FIGURES)

Introduction

The nitrogen requirements of the pineapple plant, for maximum production of fruit, have been found to vary as much as 75 per cent in successive years in essentially the same site. That the quantity of nitrogen needed may vary greatly in different areas, and in the same field in different years, is due in large part to variations in sunshine, temperature, and rainfall. Nevertheless, by methods already described there may be obtained from the growing plant in the field quantitative records that supply the information necessary for efficient use of fertilizer, regardless of location and weather. That these records are not limited in their use only to a single location or season is due to the fact that the concentration of starch in the plant is employed as an index of the net availability of CO_2 (37). Experiments were conducted to determine whether it might be of any practical importance to maintain a balanced relation between nutrients commonly added to the soil as fertilizers and the product of CO_2 nutrition. Following principles already emphasized (25, 35, 36), the nitrogen nutrition of pineapple was first considered in relation to nitrate and carbohydrate reserves (37). A very low content of nitrate was found adequate for greatest possible yields of fruit when carbohydrates were low; when starch reserves were high, maximum production was not obtained unless the plants were essentially filled to capacity with nitrate (fig. 1).

As in the case of some other monocotyledons (57, 58, 59), fertilizer applications made after blossom buds developed were without measurable influence upon yields. But about a year usually elapses from the time pineapple slips are set in the soil until blossom buds appear. During earlier vegetative stages considerable latitude may be permitted in nitrate nutrition and yet only small but significant losses in yield of fruit result. This is because a pineapple plant, low in nitrate in relation to carbohydrates, will absorb and reduce nitrate with extraordinary rapidity when a more liberal external supply of nitrate is made available. The pe-

riod of nitrate deficiency and carbohydrate accumulation, if not prolonged, is thus often balanced by the subsequent interval of new protein synthesis from nitrate, which is far more rapid than that in comparable plants lower in carbohydrates and continuously supplied with abundant nitrate.

Plants still more deficient in carbohydrates in relation to nitrate absorb and reduce nitrate even more slowly. Thus with limited nitrate reduction there is necessarily a corresponding decrease in oxidation rate of carbohydrates or their derivatives (36). Ultimately the plants thus tend to counteract the condition of carbohydrate deficiency, if subsequent weather conditions become favorable for CO_2 assimilation. These responses, however, are not peculiar to pineapple plants (36). Results in harmony with them have recently been reported by HOAGLAND and ARNON (23). For the reasons mentioned, pineapple plants in the earlier stages of vegetative growth can thus tolerate a short interval of moderate nitrate or carbohydrate deficiency without serious effect on production of fruit. But after blossom buds appear there is no further opportunity to influence nitrate reserves. Just prior to and during blossom-bud differentiation, it is essential that the nitrate content of the plant be at the proper level in relation to carbohydrates, or serious losses in yield will occur (37). The concentration of nitrate required in the plant for maximum fruit production in different locations or seasons, even at the critical period of differentiation, may vary by more than 100 per cent. It is proposed to show in this paper that potassium and phosphorus requirements are intimately associated with the required level of nitrate nutrition.

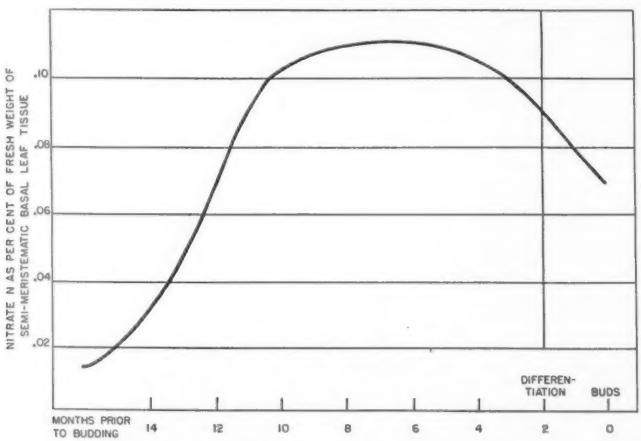


FIG. 1.—Proximate maximum percentages of leaf-nitrate N attainable at different stages of growth. Maximum nitrate is desirable only if plants contain adequate carbohydrate reserves, as indicated in warm sunny location by not less than about 15 per cent and in cool or cloudy location by about 30 per cent no. 1 yellow-green leaf color (37).

Methods

The methods employed have already been described in detail (37). The semimeristematic white basal tissues of pineapple leaves which are nearly fully ex-

panded are employed for nitrate, total potassium, and total phosphorus analyses. The tissues analyzed are comparatively homogeneous and practically free of lignified elements. They consist essentially of young parenchymatous cells, so that for all practical purposes the analytical sample is made up largely of protoplasm. The moisture content of the white basal leaf tissue fluctuates little, even though there may be a severe water deficit in specialized water-storage tissue of the more mature portion of the leaf. Regardless of environmental influences or whether the plants have been growing for 3 months or a year, exactly comparable leaves are always selected for analysis. The tissue analyzed is always semimeristematic and always from leaves of the same stage of development (**37**). These factors provide a greater degree of precision in interpretation of analytical results than do highly differentiated tissues with varying degrees of lignification and water content. Chemical analyses, particularly for phosphorus, appear to be significant in relation to yields, even when there are only small differences in content of this element in the white basal tissue of the leaves analyzed (**37**). Great differences in chemical composition among the plants under investigation would scarcely be expected, because a difference in yield of 1 ton of fruit per acre means only 0.125 pound per individual fruit when there are 16,000 plants per acre.

Although not directly comparable with the technique here employed, there is much to be said in favor of analyses of mature tissues (**5, 7, 9, 51, 52, 53**), particularly where only proximate rapid chemical methods are employed to detect the nutrient deficiencies. Relatively mobile elements, such as nitrogen (**38**) and potassium (**36**), commonly become depleted first in the older stem and leaf tissues, because of translocation from mature to meristematic cells. In consequence, lack of these nutrients is often most readily detected in older leaf or stem tissues; and if results are expressed as concentrations found in the sap or in an extract, variations in degree of lignification between unlike samples may perhaps be unimportant.

NITRATE AND CARBOHYDRATE STANDARDS.—The comparatively immature white basal leaf tissue employed for nitrate, potassium, and phosphorus analyses furnishes an excellent index of the content of these nutrients in the expressed juice of the massive stem of the plant. But starch, the major carbohydrate reserve of the vegetative organs, is not stored in quantity in the very young basal leaf tissues. Macroanalyses of the stem and of the mature portions of the leaf do not indicate to what percentage of capacity the plants may be filled with starch. This is due to the fact that relatively xeromorphic yellow-green plants often contain such a high proportion of inert lignified elements that, even though essentially all nonsenescent starch-storing parenchymatous cells are filled to capacity, expression as percentage of fresh or dry material can indicate only little starch. With respect to nitrogen nutrition, such a plant is not considered deficient in carbohydrates, even though the absolute amount may be low.

On the other hand, macroanalyses of a black-green succulent plant may indicate much more starch than in the yellow-green woody one, on an absolute-amount basis, or as percentage of fresh or dry material. Microscopic observation, however, shows that such plants are provided with only about 25 per cent of the starch their tissues are capable of holding. Such a plant is considered defi-

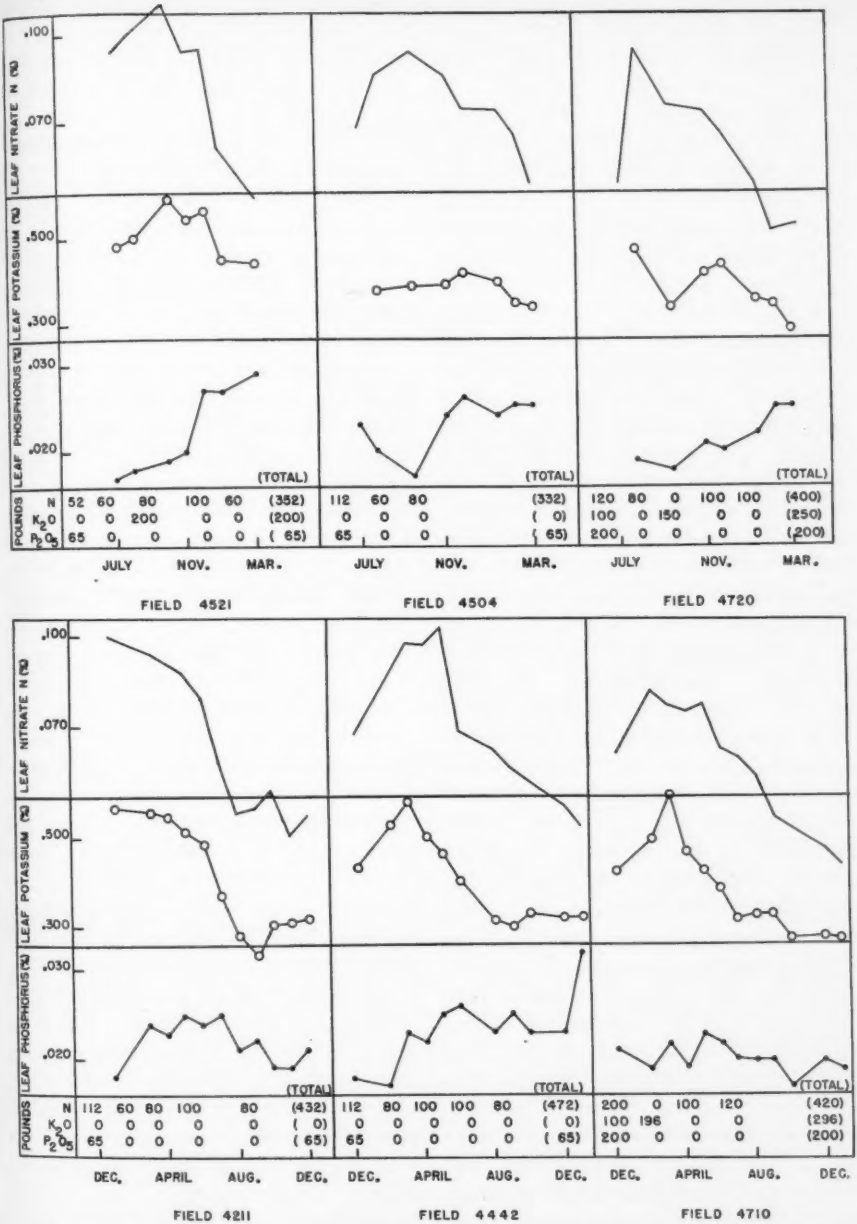
cient in carbohydrates, even though the absolute amount of starch may be higher than in the former case. Microchemical observations have shown that the degree of greenness of the leaves reflects to what percentage of capacity the plant is supplied with starch reserves. The yellower-green the plant, provided it is not senescent, the higher the starch content in the sense noted. The color standards employed for this purpose, significant within a range of plus or minus 3 per cent, are given in an earlier report (37).

Figure 1 shows the proximate maximum leaf-nitrate values attainable, but maximum nitrate reserves are desirable only if plants contain an adequate carbohydrate supply. This is indicated by about 15 per cent no. 1 yellow-green leaf color. To be conservative in the use of nitrogenous fertilizer in a location where past sunshine records indicate a low light rate, a greater carbohydrate reserve should be permitted to accumulate (about 30 per cent no. 1 color) before additional nitrogen is applied.

To prevent serious losses in yield of fruit it is particularly important to avoid either nitrate or carbohydrate deficiency at time of differentiation. In contrast, during earlier months of vegetative growth, plants may become so low in nitrate that they display 30–40 per cent no. 1 color with only relatively unimportant losses in yield, provided nitrate deficiency is rather promptly corrected. In interpretation of figures 4–24 these standards should be kept in mind, for the recorded percentages of leaf-nitrate nitrogen and no. 1 color make apparent either nitrate or carbohydrate deficiency where they appear as limiting factors in the experiments that follow. The percentages of leaf nitrate taken alone mean little, but extremely small differences in nitrate content become highly significant as associated with fruitfulness when considered in relation to carbohydrates (37). CHAPMAN and LIEBEG (12), in studies of the nutrition of citrus trees, recently obtained apparently similar results. They found that nitrogen content was not a reliable index of nitrogen requirements. They report "fluctuations in the dry weight yield of plants or plant parts, of nearly identical nitrogen content."

Discussion of results

Figures 2 and 3 show the characteristic relationships in nitrate, potassium, and phosphorus reserves which during a period of several years have appeared repeatedly in all similar routine records of fields over wide ranges of climatic and soil conditions. Analyses of the plants commenced soon after the root systems became established and there was noticeable growth of leaves. Analyses were discontinued at the time of differentiation of flower buds or shortly thereafter. The nitrate levels in the plants were much higher in some fields than in others. As already explained (37), the percentage of nitrate needed for maximum production varies with opportunity for carbohydrate accumulation. In none of these fields was nitrate a factor materially limiting production of fruit at the levels of available potassium and phosphorus. Carbohydrate synthesis, of course, depends on sunshine, temperature, and other factors which varied greatly among the areas in question. Although records of sunshine, temperature, rainfall, and soil composition are available, they are in general omitted from this discussion. They are peculiar to the site in question and often characteristic of it only for the season concerned. In contrast, quantitative plant records furnish an integration of the most complex environmental pattern



FIGS. 2, 3.—Leaf analysis in fields indicated, first taken about 3 months after planting and continued until early stages of blossom-bud development, showing intimate relationship between nitrate and potassium curves, except in field 4504 where no potash was applied and soil was very low in this element. Note typical inverse relationship between nitrate and phosphorus curves, except in fields 4211 and 4710 where available external phosphorus supply was extremely limited.

and supply information employable under a wide variety of conditions.

The absorption of anions such as nitrate is frequently greatly accelerated by the presence of rapidly absorbable cations such as potassium (**1, 2, 8, 13, 19, 26, 32, 33, 51**), and under field conditions nitrate is not absorbed freely when the potassium supply is low. Absorption of the ammonium ion is not favored by potassium (**2, 15, 35, 36**). But, regardless of the form of the nitrogenous fertilizer applied, under field conditions nitrate is almost the sole external source of nitrogen. Exceptions would be found where soil has recently been wholly or partially sterilized and in flooded areas, as in the case of rice paddies. In both cases oxidation of reduced forms of nitrogen to nitrate might be limited (**36**). As an agricultural procedure, however, potash is now applied to pineapple fields with (in part) the specific objective of increasing the nitrate reserves in the plant. There must be an external supply of nitrate, of course, but heavy additions of nitrogen to the soil are often associated with low nitrate reserves in the plant unless coupled with an adequate supply of potassium. The typical, closely corresponding curves for leaf nitrate and leaf potassium are shown in figures 2 and 3. There is not perfect correlation in all cases between leaf values for nitrate and potassium, nor would it be expected. Other cations undoubtedly play an important role. This would be true perhaps of such a field as 4504 (fig. 2), which was extremely low in available soil potassium and received no potash applications. Under weather conditions favoring carbohydrate accumulation, high leaf-nitrate values are required (**37**), as in the earlier stages of growth of field 4521 (fig. 2). But in order that the needed nitrate may be absorbed, a relatively great supply of potassium is essential. Potassium has other functions than merely accelerating the absorption of nitrate, and other cations often favor the intake of anions (**18, 22, 23, 44, 46**); but under a great variety of soil conditions there seems no practical substitute for potassium in its relationship to absorption of nitrate by the pineapple plant. On the other hand, where carbohydrate content is limited because of weather conditions, and in consequence only a low nitrate reserve is required, a leaf-potassium percentage which does not fall much below 0.380 seems adequate for other functions of potassium.

These relationships will not necessarily be directly displayed by all species of plants under all circumstances, as pointed out by HOAGLAND (**22**) and emphasized by BECKENBACH and others (**4**). For instance, under conditions of extreme potassium deficiency, plants of a type that store nitrate may ultimately become filled to capacity with it, particularly where sodium is substituted for potassium. This is probably not because of high rate of absorption of nitrate, for the plants increase in volume very slowly, but because of limited use of nitrate in new protein synthesis (**24, 38**). The potassium requirements of species that store nitrate in quantity may well be different from those of plants rarely containing more than traces (**36**). The only cation considered in these experiments is potassium, yet experiments with solution cultures and field tests have shown that high levels of potassium nutrition may be associated with calcium deficiency. Conversely, heavy applications of calcium often result in lack of sufficient potassium in the plant (**6, 14, 16, 17, 22, 29, 33, 41, 54**).

Nevertheless, under field conditions the pineapple plant apparently demon-

strates the allied absorption of nitrate and potassium. The commonly found inverse relationship between intake of two ions of like sign is indicated in figure 2 by the curves for leaf phosphorus and nitrate. This relationship will not always find expression, if there is a severely limited supply of available phosphate in the soil, as in some of the fields shown in figure 3. Many workers have found that, by decreasing the concentration of nitrate, plants may often be induced to accumulate more phosphate. Conversely, a high level of phosphate nutrition is sometimes associated with nitrate deficiency (**1, 2, 10, 12**). Under field conditions in the case of most crops, however, relatively heavy applications of phosphate are required before absorption of nitrate is noticeably depressed. On the other hand, any superphosphate or nitrogen applied to pineapple fields after planting is commonly not distributed in the soil around the plant but is placed in a localized zone—in the axils of one or two of the lower leaves (**37**). This may lead to a greater suppressing action of phosphate on absorption of nitrate than might otherwise occur, although the limiting effect of nitrate on intake of phosphate is more commonly in evidence. Apparently when climatic conditions favor carbohydrate accumulation and a high nitrate reserve is accordingly required (**37**), absorption of phosphate may be suppressed. In consequence, more liberal applications of phosphate might be needed than if the light rate were lower (and the needed nitrate therefore less), as in another season in the same site.

During earlier vegetative stages a leaf-phosphorus content of 0.020 per cent or somewhat less may be adequate. With diminution in nitrate reserves just prior to and during blossum-bud differentiation, phosphorus tends to increase in the great majority of cases, unless the available supply in the soil is extremely low. At that time, for greatest possible yield leaf phosphorus should be about 0.028 per cent, provided there are no other limiting factors, such as nitrate deficiency. When other deficiencies are corrected severe losses in fruit production occur when phosphorus reserves are below this level at differentiation. Until recently, phosphate applications just prior to differentiation were not considered feasible commercial procedure, for repeated experiments gave contradictory results. These earlier experiments in conjunction with soil analyses, however, did not include any records of the plants except yields. Fruit weights from each of six replicates were taken separately. But statistical analysis showed in some seasons highly significant gains and in others significant losses from phosphate applications just prior to differentiation. The data that follow appear to furnish an explanation. For example, in the case of field 4521 (fig. 2) leaf phosphorus at differentiation in March was at the desired value of 0.028 per cent. If a late winter application of phosphate were made to this field, it might suppress nitrate absorption, resulting in deficiency of this nutrient in relation to carbohydrates. In consequence there would probably be a decrease in production of fruit (**37**). Field 4211 (fig. 3) presents a contrasting situation. There phosphorus reserves were very low at differentiation, and added phosphate would doubtless have increased yields greatly, provided the external supply of nitrate and potassium was maintained at a level high enough to prevent their serious deficiency.

Unquestionably other anions also play a significant part and may be limiting factors in the culture of the pineapple

plant. In fields such as 4710, for instance (fig. 3), where there is comparatively heavy rainfall, considerable quantities of sodium chloride may pass through the soil. In some fields as much as 500 pounds per acre per year, owing to ocean spray, are transported by the wind and carried to the soil by rain. Chloride and other anions have been reported to suppress absorption of phosphate and nitrate (**12, 23, 30, 33, 60**), and chloride may well have been a factor in field 4710, together with sodium, which would not be expected to favor intake of potassium. As information is lacking on many essential as well as nonessential ions absorbed by the plants in these experiments, complete interpretation of results is not attempted. Also, intact plants can never furnish measurements of absorption that are distinct from metabolic activities and growth. The results of more precise studies of absorption, conducted by STEWARD (**49**) and others (**18, 44, 46**), appear in many cases to supply logical explanations of some of the responses here cited.

The quantitative chemical and physical records (**37**) to be discussed were taken on plants in plots that received various fertilizer treatments with ammonium sulphate, potassium sulphate, and superphosphate (figs. 4–24). The experiments were of the usual agronomic type. with six replications of each treatment. These plant records are informative and are presented in part in the following pages. These results have since made possible the installation of field experiments wherein fertilizer applications were not arbitrarily predetermined but were made on the basis of plant composition, past weather records for the site concerned, and absorptive capacity of prevailing root systems. The objective in these newer tests is to learn how to attain efficiently, at different stages of growth, desired concentrations of carbohydrates, nitrate, potassium, and phosphorus, and their relationship to production of fruit. The growth responses of plants in these later experiments have furnished striking evidence that the previous conclusions are essentially correct. However, fruit from these later tests will not be ready for harvesting for some months. Whether or not fruit weights may then be obtained under present conditions is uncertain.

EXPERIMENT 55-C; FIELD 4705.—This field was planted in April, 1939. Records of the plants were first taken about 6 months later and were continued until March, 1940, when blossom-bud differentiation occurred. All treatments (figs. 4–9) except the K plots had a fairly liberal reserve of nitrate in relation to carbohydrates. This is shown by the percentage of no. 1 leaf color, which at no time was much greater than 15 per cent, the arbitrary point below which carbohydrate deficiency is indicated (**37**). Thus in all plots except the K series (fig. 4) neither nitrate nor carbohydrate deficiency limited production of fruit; that is, higher levels of nitrogen nutrition would not have increased yields under the prevailing climatic conditions and at the levels of potassium and phosphorus nutrition imposed.

Correlated with relatively low leaf potassium, the A plots were consistently lower in content of nitrate than the D (fig. 5). This is not unexpected, for potassium and nitrate absorption are commonly found intimately allied in many kinds of plants (**1, 2, 8, 15, 22, 26, 28, 32, 33, 36**). The relatively low level of nitrate in A, however, was not associated with greater carbohydrate accumulation than in D, as would be anticipated if carbohydrate synthesis had occurred at an equal rate in both series. Rather, no. 1

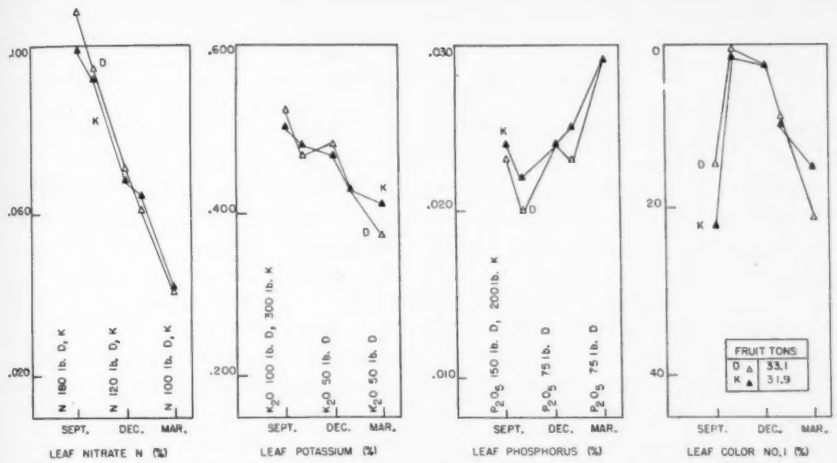


FIG. 4.—Experiment 55C, field 4705. Associated with extreme nitrate deficiency during first 6 months of growth, prior to obtaining formal records in September, are lower yields of K plots. In September, even after 6 months, note much lower nitrate and higher no. 1 color (higher carbohydrates) in K than in D plants. Leaf potassium and leaf phosphorus adequate in both groups. During early months high total concentration of fertilizers and high phosphate probably limited nitrate absorption by K plants (39).

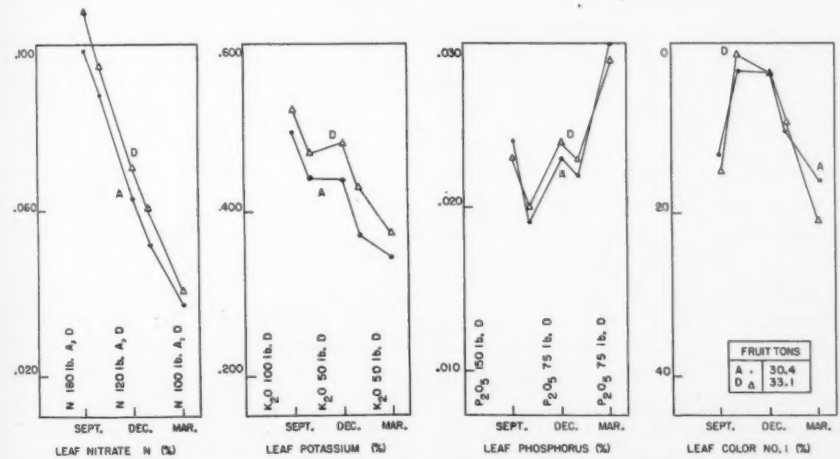


FIG. 5.—Experiment 55C, field 4705. Leaf phosphorus adequate in both groups and as high in A with no phosphorus fertilization as in D with added superphosphate. With lower leaf nitrate in A, note apparently freer absorption of phosphate (cf. fig. 6). Correlated with greater absorption of potassium in D than in A is consistently higher leaf nitrate. Nitrate proximately adequate in relation to carbohydrates, as in both A and D plots; no. 1 color not materially over 15 per cent, below which carbohydrates limit fruit production, not nitrate. Higher leaf potassium and leaf nitrate in D than A but no lower carbohydrate reserve (no. 1 color); hence larger plants and greater yields in D.

leaf color (indicative of carbohydrate content) was about the same in both until differentiation, when it was a little higher in D (fig. 5). That the lower concentration of nitrate in A was adequate in relation to carbohydrates and did not result in accumulation of a carbohydrate reserve large in relation to available nitrate may well have been due to the fact that the plants were limited in CO_2 assimilation by their relatively low content of potassium, an element which has repeatedly been found to be—directly or indirectly—essential for carbohydrate synthesis (3, 18, 24, 27, 38, 40, 42, 44, 46, 47, 48, 49, 56). In contrast, the D groups—with greater potassium supply and on that account a presumably more rapid photosynthetic rate—were apparently able to accumulate carbohydrates in approximately the same concentration as in A. This occurred even though growth of the D plants was greater and their nitrate reserves materially higher than in A. It would seem to follow that the use of carbohydrates in new protein synthesis from nitrate, known to be facilitated by potassium (8, 18, 24, 32, 38, 42, 44, 46, 47, 48, 49), while obviously greater in D than in A (as shown in part by greater growth), was compensated for by more efficient photosynthesis in the former case. The net results were much larger plants and nearly 3 tons more fruit per acre in the D than in the A plots. This was true, even though the concentration of carbohydrates and probably organic nitrogen was much the same in both groups of plants and the quality (not the size) of their fruit very similar, at least with respect to such factors as flesh texture and color and percentage content of sugars and total acids.

Thus, although the leaf-nitrate values were not the same in all plots, they were adequate in relation to carbohydrates (except in the K group) during the early stages of growth. In this treatment very heavy applications of potassium and phosphorus during the early months of the experiment, prior to obtaining formal records, were associated with extremely yellow leaf color. This may have been due in part to typical limiting effects of a high concentration of salts on absorption and assimilation of nitrate (39), brought on by the application of relatively large amounts of fertilizer. Also, the comparatively heavy application of phosphate to the K plants may have contributed to suppression in intake of needed nitrate (1, 2, 10, 11, 12, 13, 15, 23, 30, 33, 36, 42, 50, 51, 57, 60). In any event, the net result was extreme nitrate deficiency during the first half year of growth, which was not fully corrected at 6 months after planting. Even at that time, when the first analyses were made in September, the plants of the K plots were far lower in nitrate than those of the D and much higher in carbohydrates, as shown by a materially greater percentage of no. 1 color (fig. 4). The K series were equal to or higher than the D in potassium and phosphorus content but were outyielded by the D, undoubtedly because of nitrate deficiency in relation to carbohydrates, which was far more severe during the early months than is indicated by the first plant records taken in September.

In experiment 55-C, field 4705, for odds of 19:1 there is required a difference in yield between any two treatments of 0.83 tons of fruit per acre. As shown in figure 6, the B plots outyielded the A by 1.5 tons. The lower nitrate content of the A plants was not a limiting factor, for their carbohydrate reserves were too low to warrant a higher concentration of nitrate, as shown by the fact that at all times no. 1 color was 15 per cent or lower (37). Neither was phosphorus deficient

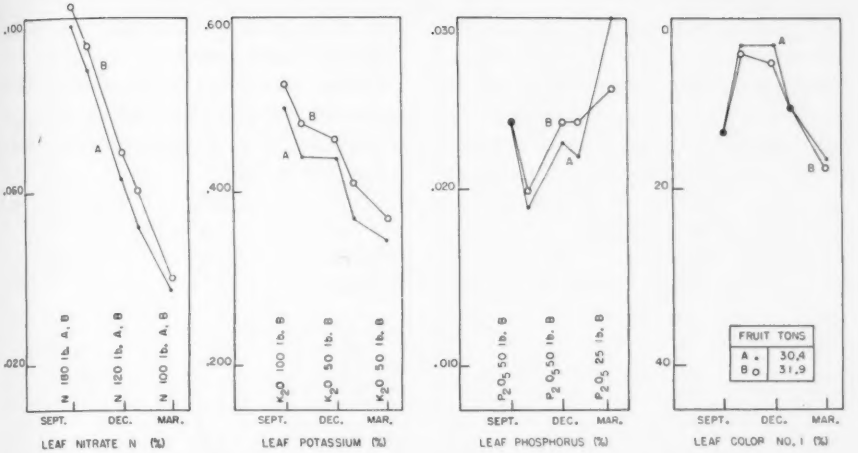


FIG. 6.—Experiment 55C, field 4705. At differentiation in March, higher leaf phosphorus in A than B, though only latter fertilized with phosphorus. Lower nitrate in A associated with freer absorption of phosphate. With same nitrogen fertilization, greater potassium absorption by B, correlated with consistently greater intake of nitrate; but B no lower than A in carbohydrates (no. 1 color). Potassium deficiency limited yields in A because of less absorption of nitrate and apparently lower plane of photosynthetic activity than in B.

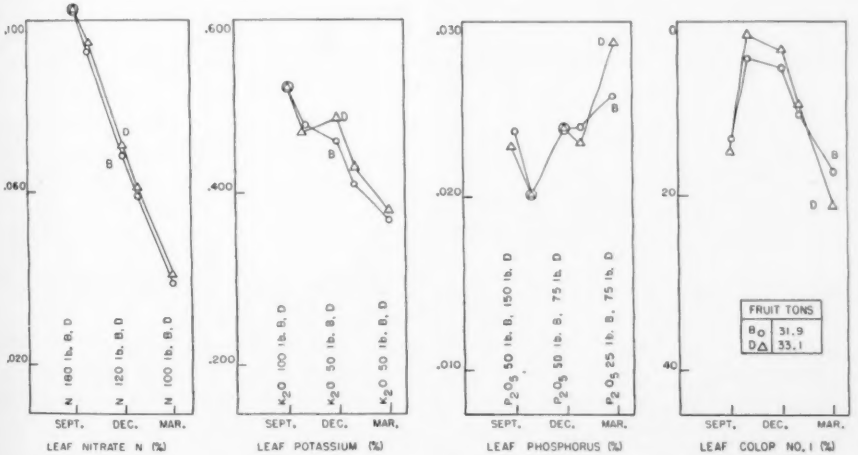


FIG. 7.—Experiment 55C, field 4705. D plots outyielded B because at critical period of differentiation in March D was at desired level of 0.028 per cent leaf phosphorus, with B definitely deficient. Essentially no difference in nitrate concentration between treatments but slightly higher potassium content of D may have contributed to significant but only slightly higher carbohydrate reserves (greater no. 1 color) in March. This might advantageously have been coupled with slightly higher leaf nitrate.

in A, even though no phosphate was added to the plants of this series. In fact, coupled with lower nitrate than in B, and presumably in consequence less suppressing action on phosphate intake, it was absorbed more freely by the A plants during the critical period of differentiation in March than by the plants of the B plots. Probably there occurred for this reason a leaf-phosphorus value of 0.030 per cent, much higher than in B, even though only the latter was fertilized with superphosphate. Clearly the B plots were more productive than the A, owing to their higher content of potassium, as shown by the leaf analyses for this element (fig. 6). Coupled with higher potassium in the B plants was a higher reserve of nitrate than in A. This invariably seems to occur in pineapple plants under field conditions, when there is available an adequate external supply of nitrate in the soil. But although the concentration of nitrate in the B plants was relatively high, their carbohydrate content, as shown by percentage of no. 1 color, was no lower than in the A plots—a situation already discussed in connection with figure 5.

But the B plots were in turn outyielded by the D group that produced 1.2 tons more fruit per acre. Reserves of nitrate, potassium, and carbohydrates, the latter indicated by no. 1 color, were much the same in both series (fig. 7), the most important difference being in the leaf-phosphorus content of the D plots at differentiation in March (0.029 per cent as compared with 0.026 for B). That the leaf-phosphorus value of 0.029 per cent was adequate is shown by the fact that a higher phosphorus reserve in E at differentiation (fig. 8) gave no increase in yield over D, with other determined factors essentially the same. Likewise the H plots (fig. 9), which varied from the D

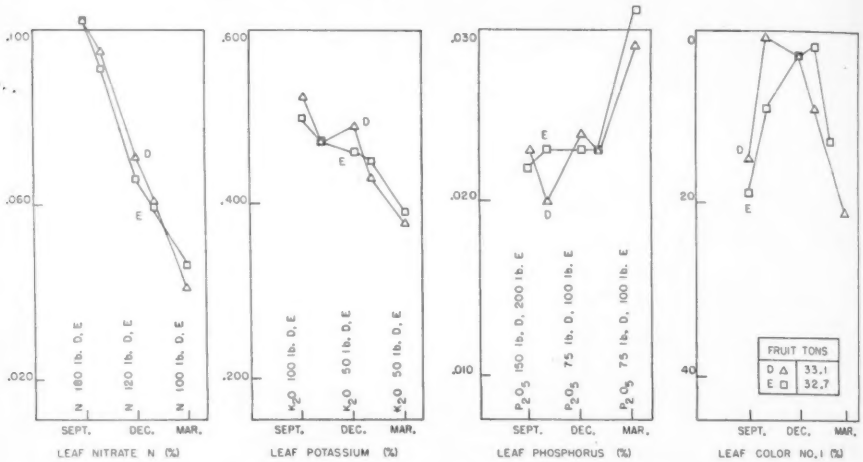


FIG. 8.—Experiment 55C, field 4705. No significant difference in yields. Leaf potassium adequate as about 0.380 per cent, or higher, at all times and similar in both treatments. In each series nitrate proximately adequate, though a little low in E plants only in September and low in D only in March, in relation to carbohydrates (no. 1 color). Leaf phosphorus in E higher than 0.028 per cent at differentiation did not increase yields.

chiefly in having a higher leaf-potassium content, indicate that potassium levels in D were adequate, for the yields of the H plots were not significantly increased over the D by their higher reserve of potassium. But the D plots at differentiation, when leaf potassium was lowest, still contained about 0.380 per cent.

For maximum yields in field 4705, under the climatic conditions prevailing, it was essential to fertilize with nitrogen, potassium, and phosphorus. In another year in the same location, however, if conditions were less favorable for carbohydrate synthesis, a lower level of nitrate nutrition would be indicated. Under these conditions it is doubtful whether phosphorus fertilization would be needed, as with a lower nitrate supply phosphate would probably be absorbed more freely, as in the plants of the A plots (fig. 6). With respect to potassium, considering the experimental results just discussed and those that follow, if in another year—because of lower carbohydrate content—lower nitrate reserves were required, less potassium would be needed for adequate intake of nitrate. In order not to limit metabolic activities such as protein and carbohydrate synthesis, however, for which potassium is directly or indirectly essential (**3, 8, 18, 24, 27, 32, 38, 40, 42, 44, 46, 47, 48, 49, 56**), where root systems and soil moisture permit, leaf potassium should probably not at any time be allowed to fall much below 0.380 per cent (figs. 7, 9), even though with a lower leaf-potassium content absorption of nitrate might be adequate in relation to low carbohydrate reserve.

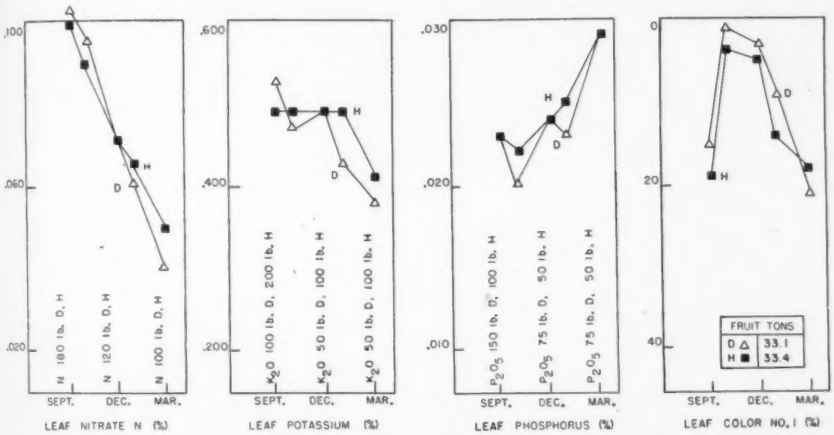


FIG. 9.—Experiment 55C, field 4705. No significant difference in yields. At differentiation in March leaf phosphorus at desired 0.028 per cent in both H and D. During vegetative period phosphorus 0.020 per cent or higher in both cases, hence not limiting factor. Nitrate in relation to carbohydrates (no. 1 leaf color) proximately adequate in both groups. Higher leaf potassium of H than D plots, December to March, did not significantly increase yields, indicating 0.380 per cent leaf potassium (as in D) adequate, where more is not needed for absorption of nitrate.

EXPERIMENT PK-3; FIELD 4703.—This field was planted in the fall of 1938, and the following spring records were taken of experimental plots until blossom-bud differentiation in December (figs. 10–13). While leaf-nitrate values were much the same in all plots and dur-

ing the spring approached the maximum concentration (fig. 1), they should have been somewhat greater, from March to June. This is indicated by the record for no. 1 leaf color, which in all plots was 40 per cent or more, indicative of high reserve of carbohydrates. However, the June application of 120 pounds of nitrogen per acre was associated with prompt and marked decrease in concentration of carbohydrate, as indicated by decrease in no. 1 color. In later months (and during differentiation in December), although leaf nitrate in all plots was much below capacity (fig. 1), it was nevertheless in every case very high in relation to carbohydrates, for the plants of all series displayed only about 10–15 per cent no. 1 color, indicative of a carbohydrate reserve on the borderline of deficiency.

The percentage of potassium in the leaves was about the same in all treatments and after June was extremely low, except in D and C in December. Potassium is apparently essential for free absorption of nitrate by the pineapple plant, as already pointed out. The low leaf-potassium values prevailing during the last half of the vegetative period were associated with a rate of absorption of nitrate which at that time was adequate in relation to carbohydrates, which were likewise extremely low. If there had been greater carbohydrate reserve, however, indicated by higher percentage of no. 1 color, and a higher nitrate content had in consequence been required, a more liberal potassium supply would probably have been essential for sufficiently rapid absorption of nitrate. Although the low potassium reserves of summer and fall, as in A and B, permitted accumulation of all the nitrate required, lack of potassium in later stages of growth was probably a factor adversely influencing new protein synthesis from nitrate and carbohydrate accumulation.

The comparatively yellow color of the

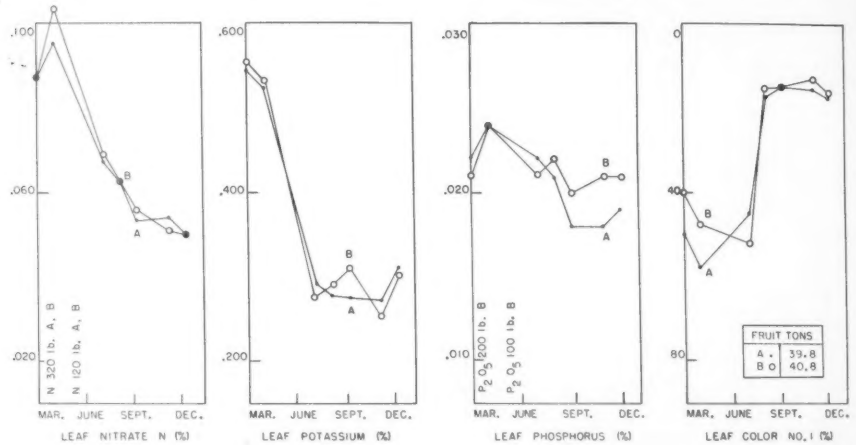


FIG. 10.—Experiment PK3, field 4703. After June, plants of both groups became extremely low in leaf potassium. During earlier months leaf nitrate too low, in relation to carbohydrates, no. 1 leaf color being about 40 per cent. Following June nitrogen applications, nitrate more than adequate, no. 1 leaf color remaining about 10 per cent. Greater yields of B correlated with inadequate but greater leaf-phosphorus values than A just prior to and during differentiation in December.

D plots from March to June (fig. 11) as compared with the others was undoubtedly a reflection of higher carbohydrate reserve, probably correlated with comparatively high soil-solute concentration, as the D plants in the earlier stages of growth received much more total fertilizer than any other group. A high salt concentration in the nutrient medium may induce carbohydrate accumulation (39). On the other hand, the greater supply of potassium and phosphorus available to D may have aided in synthesis of carbohydrates in the earlier months of growth, as already mentioned in connection with experiment 55-C in field 4705 (figs. 4–9).

With nitrate and carbohydrate reserves shown unimportant variables among treatments, except as indicated in the D plots, potassium and phosphorus may be considered in their relationship to yields, bearing in mind that there was required for odds of 19:1 a difference in yield between any two treatments of 0.94 tons of fruit per acre. As shown in figure 10, the B plots significantly outyielded those of the A. It is apparent that the potassium content in the two lots was almost the same and that the chief difference was in the greater leaf-phosphorus values of the higher-yielding B plants just prior to and during differentiation in December.

The D plots outyielded the B (fig. 11) but not enough for odds of 19:1. However, the slightly greater yield of D was correlated with slightly higher leaf-potassium values just prior to and during differentiation. In figure 12, the differences shown between treatments in leaf nitrate, leaf potassium, and leaf color (carbohydrates) were probably ineffective. This is indicated by the fact that when nitrate, only in September, was materially higher in D than in C, it was excessively high in both in relation to carbohydrates, as shown by the no. 1 leaf-color values. These were less than 15 per cent, indicating that both groups were

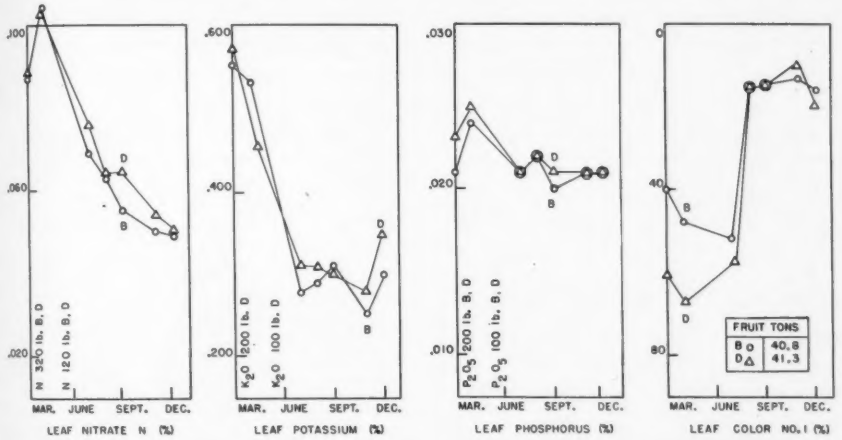


FIG. 11.—Experiment PK3, field 4703. Potash applications applied too early, for plants were then proximately filled to capacity with potassium (0.600 per cent leaf potassium). Slightly higher leaf potassium in D in winter months possibly beneficial, for D plots outyielded B, but not enough for odds of 19:1.

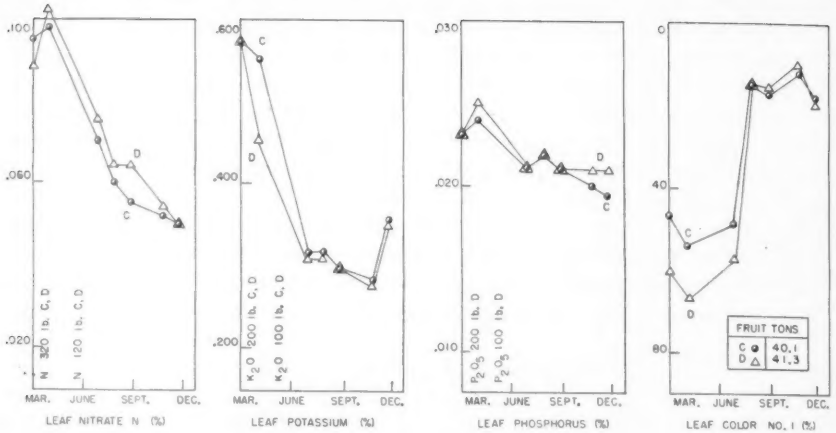


FIG. 12.—Experiment PK3, field 4703. Differences in treatments in leaf nitrate, leaf potassium, and leaf color (carbohydrates) probably largely negative in effect on yields, since when nitrate in September was significantly higher in D than C, nitrate was excessively high in both in relation to carbohydrates (no. 1 color less than 15 per cent in both). Leaf potassium higher in C only in April and then abundant in both series. D significantly outyielded C probably because of small but higher leaf phosphorus at critical period, just prior to and during differentiation in December (cf. figs. 7, 10, 13, 15).

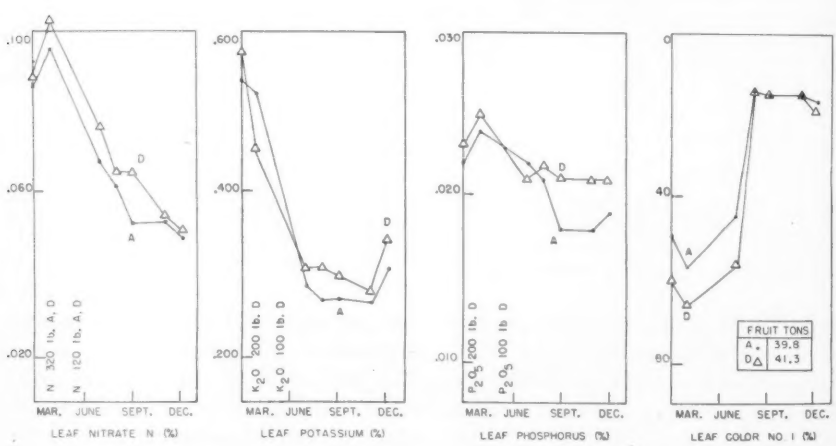


FIG. 13.—Experiment PK3, field 4703. Greater nitrate reserves in D than in A from March to June, when carbohydrates were high (no. 1 color over 40 per cent), undoubtedly contributed to increased yields. Leaf phosphorus far below desired 0.028 per cent at differentiation in December; but its much higher content in D than in A at that time probably major factor in higher tonnage of D.

on the borderline of carbohydrate deficiency, not nitrate deficiency (37). With respect to leaf potassium, it was materially higher in C than in D only in April, when there was an abundant reserve of this nutrient in both lots. The significantly higher yield of the D plots as compared with the C was probably correlated with the higher percentages of leaf phosphorus just prior to and during the critical period of blossom-bud development in December. It is recognized that the differences in phosphorus values referred to are small, but it has been demonstrated in many experiments, only a few of which can be presented in this paper, that very small deficiencies in phosphorus at differentiation have severe adverse influence on production of fruit (cf. figs. 7, 10, 13, 15). Comparison of the A and D plots in figure 13 further emphasizes the importance of adequate reserves of phosphorus at differentiation. As indicated in figures 4–9, 21, and 24, however, even the D series would probably have given much greater yields if leaf phosphorus had been up to 0.028 per cent at differentiation and leaf potassium had at no time been less than about 0.380 per cent (figs. 5, 18, 20, 22, 23, 24). During the earlier stages of growth, leaf potassium in the plants of the A and D plots was nearly up to capacity, about 0.600 per cent, even though no potash had been added. Also from March to August the A plots that received no phosphorus fertilization had approximately the same leaf-phosphorus content as the D. Effects of late applications of potassium and phosphorus would probably have been most informative. Apparently both potash and phosphate were applied at a time when neither was needed.

EXPERIMENT 55-C; FIELD 4217.—This field was planted in the fall of 1939, and records (figs. 14–17), first taken in January, 1940, were continued until blossom-bud differentiation in October. Figure 14 again emphasizes that under field conditions in Hawaii there is often indicated an intimate relationship between absorption of nitrate and potassium by the pineapple plant. Coupled with potash applications to the B plots and an increase in concentration of potassium in the plants as compared with A, the B groups were materially higher in leaf nitrate. This was true even though both series received the same nitrogen treatments. Even with much higher nitrate reserves, the B plots were not lower in carbohydrates than the A, for the percentages of no. 1 color were almost the same in both. Unquestionably the greater nitrate content of B favored more rapid utilization of carbohydrates in new synthesis of protein from nitrate, indicated in part by the greater growth and yields that the plants made. But that this did not result in lower carbohydrate reserves was perhaps correlated with favorable influences on CO_2 assimilation of the comparatively high potassium and phosphorus content of the B plots, already mentioned in the discussion of experiment 55-C in field 4705. Thus in neither A nor B was nitrate low in relation to carbohydrates, except in August, when the carbohydrate reserve was—for the stage of growth—high in both plots (about 30 per cent no. 1 color).

The phosphorus applications to the B plots materially increased leaf phosphorus over that of A, although at the critical period of differentiation in late fall both groups were extremely low in phosphorus, as compared—for instance—with the D plots in figure 7, which were up to 0.029 per cent leaf phosphorus at differentiation. The potassium and phosphorus values, materially higher in B than in A, were correlated with increase

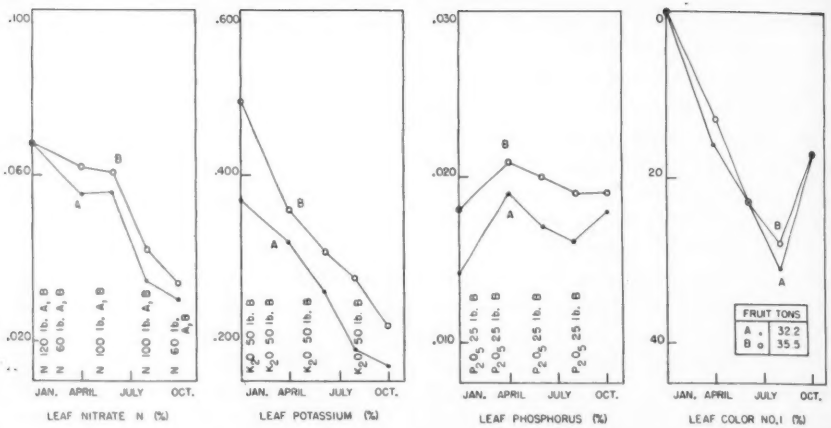


FIG. 14.—Experiment 55C, field 4217. Higher leaf potassium and greater intake of nitrate in B than in A, though both series had same nitrogen fertilization. Leaf nitrate in either series low in relation to carbohydrates (no. 1 leaf color) only in August and throughout concentration of carbohydrates about the same in both groups. Apparently higher potassium and phosphorus reserves favored greater CO_2 assimilation, as relatively high nitrate in B coupled with no lower carbohydrates, yet much larger plants and nearly 3 tons more fruit per acre, obviously required greater use of carbohydrates in new protein synthesis.

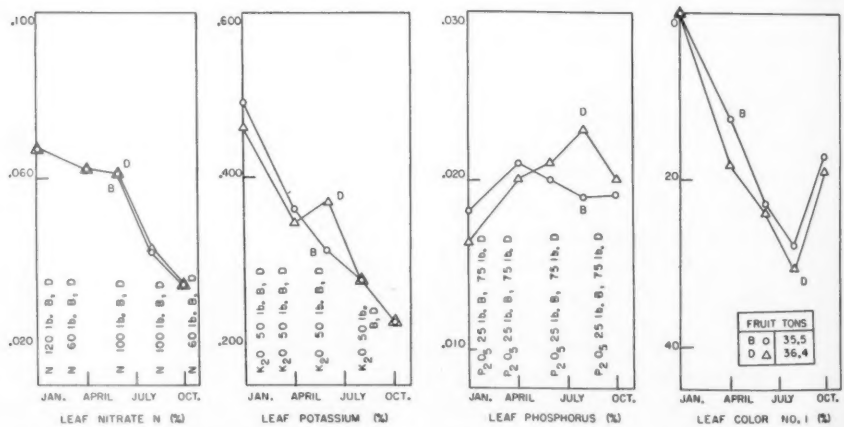


FIG. 15.—Experiment 55C, field 4217. That lower leaf phosphorus in B at differentiation in October limited production is suggested by higher phosphorus reserves in D at that time and greater yields, although differences in tonnage not great enough for odds of 19:1.

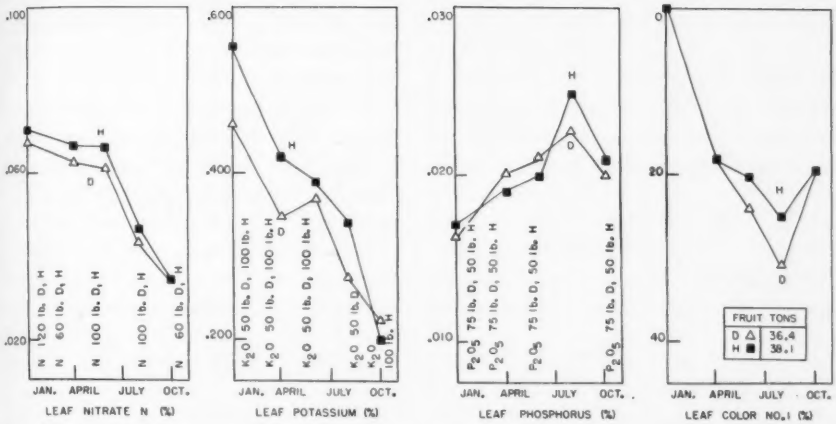


FIG. 16.—Experiment 55C, field 4217. Nitrate reserves adequate in both D and H until June, when higher potassium levels of H were coupled with greater nitrate absorption. In only D in midsummer was nitrate seriously inadequate in relation to carbohydrates, for then no. 1 leaf color was over 30 per cent. More favorable nitrate reserves in H, with more nearly adequate phosphorus at differentiation, contributed to higher yields than in D.

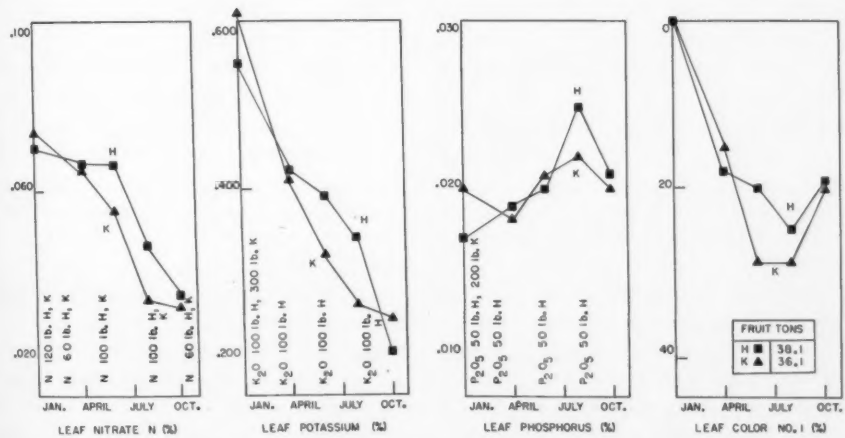


FIG. 17.—Experiment 55C, field 4217. From May to August K plots deficient in nitrate in relation to high no. 1 leaf color. Higher potassium reserves in H coupled with greater intake of nitrate than in K. Higher leaf phosphorus in H just prior to differentiation in October undoubtedly contributed also to 2 tons greater yield than in K.

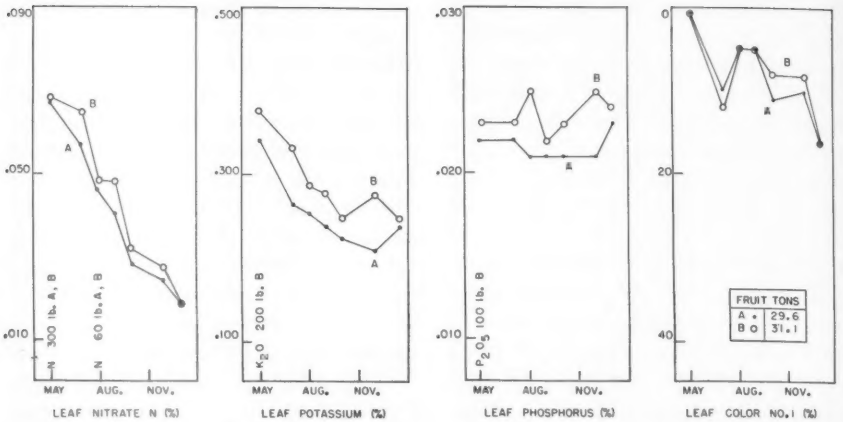


FIG. 19.—Experiment 55B, field 4710. With same nitrogen fertilization, higher potassium content of B plants was correlated with apparently slightly higher nitrate reserves, but carbohydrates were about the same in both A and B, and relatively low at all times (no. 1 leaf color 15 per cent or less). Clearly 1.5 tons more fruit per acre in B than A was associated with higher leaf potassium and possibly leaf phosphorus, though at critical period of differentiation in January A was nearly as high as B in phosphorus. Earlier higher phosphorus reserves in B were probably ineffective (cf. figs. 21, 24).

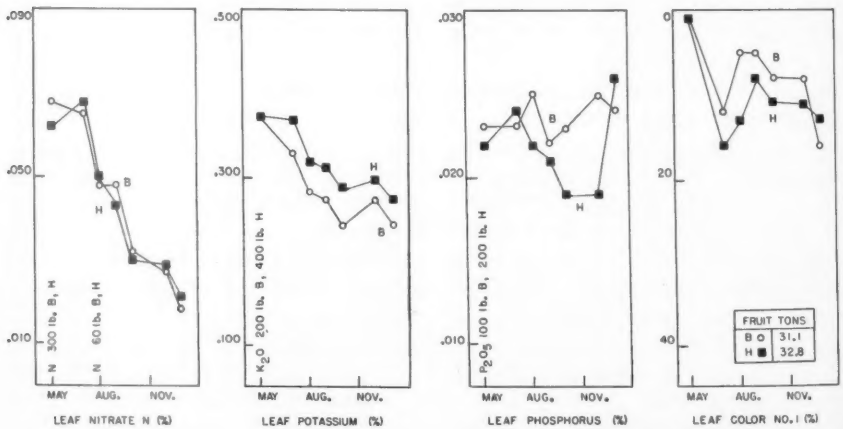


FIG. 20.—Experiment 55B, field 4710. Nitrate about the same and adequate in both B and H plants, as carbohydrates were low, not nitrate (no. 1 leaf color 15 per cent or less at all times). Higher potassium reserves in H brought increased yields over B and with higher potassium intake; carbohydrates, although low, were higher in H, July to December. At differentiation in January, leaf phosphorus, slightly higher in H, was probably advantageous.

seemingly fails to demonstrate potential possibilities in production of fruit for the season concerned in field 4710, chiefly because leaf-potassium values, while adequate for absorption of all required nitrate, should presumably have been not less than 0.380 per cent (figs. 4–9, 18, 20, 22, 23, 24). With more potassium available in later stages of growth there might have been accumulation of a carbohydrates than the B, indicated by a greater percentage of no. 1 color. It is highly probable that the potassium reserves, which were very low—even in the H plots—after August, materially limited synthesis of carbohydrates. Nevertheless, because absorption of nitrate was apparently also limited by lack of potassium, there resulted a condition of perhaps proximate balance between nitrate

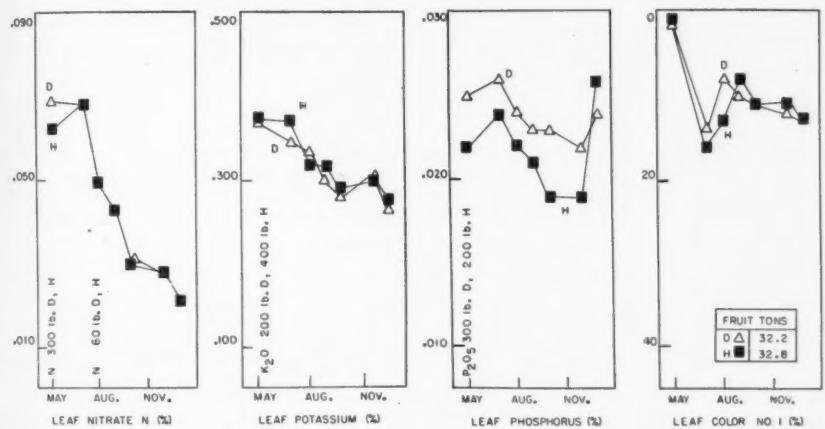


FIG. 21.—Experiment 55B, field 4710. With almost the same potassium reserves there was no essential difference in nitrate or in carbohydrates (no. 1 leaf color) between D and H plants. Relatively high leaf-phosphorus values in D only during vegetative growth (May to December) were ineffective, yields being not significantly different between treatments; but at differentiation, H was slightly higher in leaf phosphorus (cf. figs. 7, 10, 13, 15).

greater reserve of carbohydrates, which would have made desirable a greater supply of nitrate. This in turn might have made necessary more liberal applications of phosphate, in part because of the greater suppressing action that additional nitrate might have on phosphate absorption, already discussed.

Even the highest-yielding H plots (fig. 20) seemingly displayed a condition of proximately balanced multiple deficiencies (**43, 51, 52, 53**). For example, correlated with higher potassium content the plants of the H plots were higher in (and presumably organic nitrogen) and carbohydrates, with both on a comparatively low plane. On the other hand, the low level of nitrate nutrition doubtless favored absorption of the phosphate ion (fig. 2); even the A plants, unfertilized with phosphorus, absorbed this element rather freely, particularly at differentiation, when nitrate reserves were lowest (fig. 19). Commonly, pineapple plants with preconceived differential fertilizer treatments of the type indicated in the foregoing experiments produce fruit in any one site which, although varying

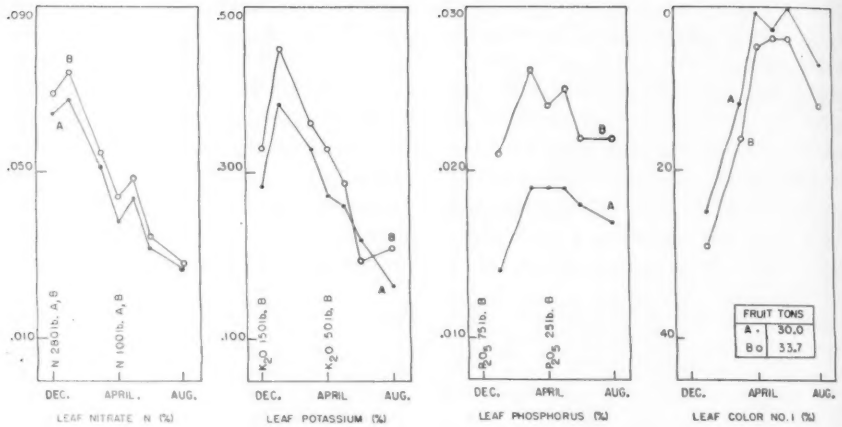


FIG. 22.—Experiment 55B, field 4720. Minor differences, though in right direction, are the higher potassium, nitrate, and carbohydrate reserves of B over A. Chief factor for 3.7 tons more fruit in B than A plots was higher phosphorus content. Leaf phosphorus unnecessarily high in B, from March to June, but should have been up to 0.028 per cent at differentiation in August (cf. figs. 7, 10, 13, 15, 24).

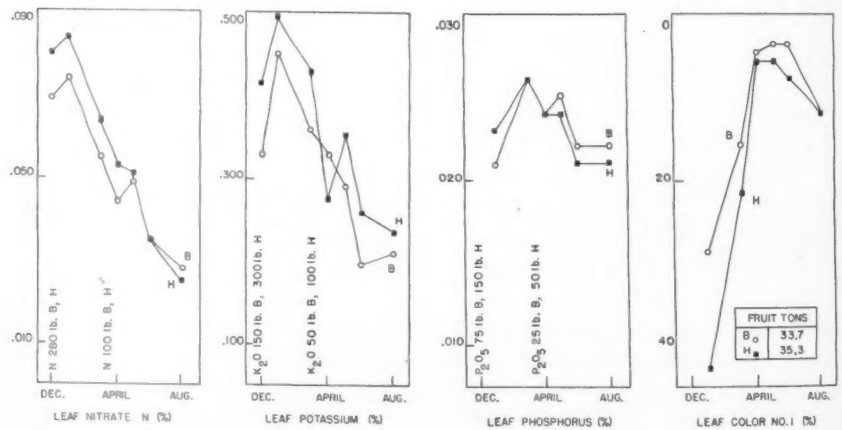


FIG. 23.—Experiment 55B, field 4720. That additional potassium as well as phosphorus was needed in field 4720 is indicated by increase in yield of 1.6 tons for H as compared with B. Except in April, leaf potassium was higher in H than B and induced higher nitrate early. High nitrate was then needed, for carbohydrate reserve (no. 1 leaf color) was relatively high in H. These factors apparently overbalanced favorable influences of slightly higher leaf phosphorus in B than in H at differentiation in August.

greatly in size among treatments, is—if harvested on the same date—often of about the same quality with respect to such factors as flesh color and texture, sugars, and acidity.

The interplay of compensating relationships in absorption and metabolism just mentioned would seem to suggest the reason for the little difference in fruit quality. These observations may be pertinent in relation to recent reports by MAYNARD (31) and HAMNER *et al.* (20) on the nutritional value of agricultural crops with various prescribed fertilizer treatments. For example, concerning ascorbic acid in tomato fruits, the latter workers conclude that "a macronutrient supply adequate to produce a fair yield is not a limiting factor in ascorbic-acid content." This seems to be in harmony with the responses of pineapple plants just described. Also they report that some of the tomato plants grown out-of-doors in sand culture produced fruits significantly higher in ascorbic-acid content than those grown in soil (20).

EXPERIMENT 55-B; FIELD 4720.—This field was planted in the early summer of 1938. Records were first taken in December and were continued through the period of blossom-bud differentiation in August (figs. 22, 23). Figure 22 shows that the chief factor for increase in fruit production of 3.7 tons per acre of B over A was the higher phosphorus content of the former group. Both the B and H plots had about the same leaf-phosphorus content (fig. 23); but that potassium as well as phosphorus was seriously deficient in field 4720 was evident from the significant increase in yield of 1.6 tons for H as compared with B, 1.5 tons being required for odds of 19:1. This gain was apparently correlated mainly with the leaf-potassium values in the H plots, which—except in April—were consistently much higher than in B (fig. 23). The higher potassium intake of the H plants for the same period was coupled with greater absorption of nitrate, until probably the external supply of nitrogen became very low in late summer, since no nitrogenous fertilizer was applied after March. Nevertheless, even in the H series nitrate reserves in relation to carbohydrates were very low prior to April. Carbohydrates during that time, as indicated by no. 1 color, were comparatively high in H, very likely owing in part to a favorable effect on carbohydrate synthesis of the relatively more liberal potassium reserves (figs. 5, 18, 20, 24). After June, with leaf potassium well below 0.300 per cent and a desirable concentration probably about 0.380 per cent, there was rapid decrease in carbohydrates, as indicated by the low percentage of no. 1 color in all treatments, so that if more nitrogen had been added to the plants in the later months it would have been largely ineffective even if absorbed (37).

Higher reserves of both potassium and phosphorus would evidently have been required during the summer months for maximum production. And if both potassium and phosphorus were applied and absorbed, with possibly an increase in carbohydrate synthesis resulting, heavier nitrogen fertilization might have been necessary to maintain adequate nitrate reserves and to obtain material benefit from additional fertilization with the other elements mentioned.

EXPERIMENT PK-3; FIELD 4817.—Records taken for a brief period were terminated at about the time of blossom-bud differentiation in April (fig. 24). Fertilizer applications gave negative results as far as yields were concerned, but the plant records are of considerable practical interest, in that in relation to produc-

tion of fruit they serve further to establish desirable levels of leaf potassium and of leaf phosphorus. While nitrate was not high as compared with the maximum values shown in figure 1, it was more than adequate; for carbohydrates, particularly at the critical period of differentiation, were low (only about 5 per cent no. 1 color in April). Accompanying greater intake of potassium by the plants proximately up to the desired level of 0.380 per cent.

There is some suggestion that the higher potassium—and perhaps phosphorus—content of the D plants was correlated with greater carbohydrate accumulation than in A, as shown by the somewhat higher percentage of no. 1 color of the D plants; but carbohydrate synthesis, if higher in D than in A, did not provide a

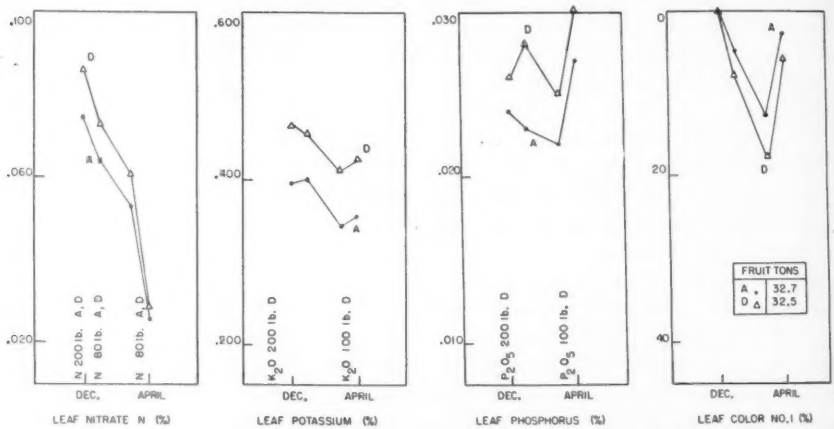


FIG. 24.—Experiment PK3, field 4817. Nitrate not a limiting factor in A or D, for carbohydrates were low (no. 1 color 15 per cent or less at all times). Much higher leaf potassium in D than in A gave no increase in yield; but leaf potassium in A not materially less than 0.380 per cent. With low carbohydrate content, potassium reserves of A were adequate for absorption of needed nitrate. At differentiation in April leaf phosphorus in A up to nearly 0.028 per cent and during vegetative period above 0.020 per cent, hence higher phosphorus content of D ineffective.

of the D plots, nitrate as usual was relatively high, but unnecessarily high in relation to the low carbohydrate content. Failure of the plants to accumulate a more liberal carbohydrate reserve would not be unexpected, however, as field 4817 is in a cloudy cool area with relatively heavy rainfall. Thus the higher nitrate reserve of D did not increase fruit production over A (37). The A series, which received no potassium fertilization, were also probably high enough in content of this element, for even at differentiation in early spring leaf potassium was ap-

high enough reserve to warrant greater leaf-nitrate values than prevailed. As indicated in other experiments (figs. 5, 18, 20, 22, 23), it appears unnecessary to maintain a higher leaf-potassium content than 0.380 per cent, except where needed to facilitate absorption of nitrate, as when carbohydrates are high (37).

The phosphorus applications to the D plots during the months of vegetative development resulted in a striking increase in reserves of this element (fig. 24). At differentiation, however, even in the A plots (unfertilized with this element) leaf

phosphorus was 0.027 per cent—practically at the desired arbitrary value of 0.028 per cent (figs. 7, 8, 21). During earlier stages of growth the phosphorus content of A was much less than that of D, but at all times it was well above 0.020 per cent. There seems to be nothing to indicate that leaf-phosphorus values higher than 0.020 per cent are necessary during the vegetative period (figs. 4, 8, 15, 16, 17, 21, 24), provided leaf phosphorus reaches 0.028 per cent at differentiation. It may again be emphasized that in cases where both nitrate and phosphorus are below desired levels, correction of either alone might well bring about still further deficiency of the other, owing to possible competition for absorption between nitrate and phosphate (fig. 2).

Although the fertilizer treatments are clearly reflected in the leaf analyses, it seems obvious that neither potassium nor phosphorus was the limiting factor in field 4817. Under different seasonal conditions, however, even in the same location, if conditions were more favorable for carbohydrate synthesis higher levels of nitrate would be required, and probably a more abundant supply of potassium would be needed, for adequate absorption of nitrate. In turn, because of the additional nitrate, there would be expected a greater suppressing action on absorption of phosphate, with possible need for phosphorus fertilization.

Because of space limitation, additional figures illustrating other results are not given; those included represent but a fraction of the data which might be presented. While the plant responses have varied in pattern in different locations and in different years in essentially the same site, in no case have responses been obtained which have appeared to be aberrant in relation to those presented. However, situations have occurred that are not illustrated in the figures shown. While not of common occurrence, the following in particular would seem to deserve mention, even though further data will be required before drawing final conclusions. In experimental plots in certain fields in some years the addition of only nitrogen and potassium has given substantial increases in fruit production. In other plots in the same experiment, the application of only nitrogen and phosphate has given comparable gains in yield. But with the same nitrogen supply, treatment with both potassium and phosphate has in some cases given no greater increases in tonnage than either alone.

In cases where potassium has been the chief factor limiting yields, additions of it should increase fruit production. On the other hand, studies of interrelationships of nutrient ions (**18, 42, 44, 46, 49**) have demonstrated that phosphate (as well as nitrate) facilitates absorption of potassium. Phosphate fertilization, therefore, even though not directly needed, might induce greater potassium absorption, and sometimes does, as indicated by leaf analyses of pineapple plants not reported here. Of course the reverse situation might possibly occur, as potassium would be expected to contribute to greater absorption of phosphate and nitrate ions. With free intake of the latter, however, phosphate absorption would undoubtedly be severely repressed, at least in pineapple. There is considerable precedent for the foregoing (**13, 15**), and a report by LILLELAND and BROWN (**28**), working with peach trees, seems pertinent. They state in part, "potassium was increased in the leaf by applications of phosphorus. . . . . Growth increases in 1938 and 1939 could be correlated with potassium (in the leaves), although no potassium had ever been added."

There were only comparatively small

differences in time of blossom-bud differentiation and fruiting among the respective treatments in the experiments discussed here. Such experiments were chosen for presentation because, if any material difference had occurred in treatments in length of time available for vegetative growth, it would have been a dominant factor in determining the quantity of fruit produced. Pineapple plants with a relatively long period of vegetative expansion will almost invariably far outyield others in the same site with a shorter interval, except under extremely adverse nutritional or climatological conditions. Thus it would have been difficult, if not impossible, to have made direct comparisons of one fertilizer treatment with another in relation to plant composition and yields, if the respective plots within an experiment had varied greatly in time of fruiting.

FIG. 25.—A plots (left): 370 lb. nitrogen, 120 lb. K_2O, and 185 lb. P_2O_5 applied per acre. C plots (right): 500 lb. nitrogen, 500 lb. K_2O, and 300 lb. P_2O_5 applied per acre. Essentially all plants in A plots display fruit, as compared with relatively few in C. Nitrate in relation to carbohydrates, prior to and during bud development, was low in A and relatively high in C plants.

As brought out earlier (37), during seasons when minimum temperatures are not low, plants deficient in nitrate and high in carbohydrates may fruit months earlier than others containing a more liberal reserve of nitrate but a lower concentration of carbohydrates. Nevertheless, plants apparently differentiate flower primordia simultaneously, regardless of material differences in nitrate or carbohydrates, provided minimum temperatures are sufficiently low (37). Figure 25, however, illustrates fertilizer plots wherein time of fruiting was in large part dominated, directly or indirectly, by available nutrient supply. These plots were planted on the same day, but the plants were widely different in time of blossom-bud differentiation and fruiting. The A plants, most of which fruited early, had at the initial stages of bud development 0.043 per cent leaf-nitrate nitrogen and a liberal reserve of carbohydrates (22 per cent no. 1 color). In contrast, the C plants, with relatively few displaying blossom buds, at that time contained 0.062 per cent leaf-nitrate nitrogen but only 11 per cent no. 1 leaf color. The latter symptom was in part an external expression of a relatively low concentration of carbohydrates (37). Comparable differences in nitrate and carbohydrate reserves between A and C were apparent weeks before the earliest stages of differentiation in the A plants.

The greater nitrate reserves of the late-fruiting C plots were associated not only with relatively liberal fertilization with nitrogen but with greater potash supply, as compared with A (fig. 25). Nitrate is not freely absorbed when potassium is low. This was apparent in bordering experiments, in the same field and planted

at the same time, where with low potash fertilization marked differences in nitrogen applications were without material influence on time of fruiting. The relatively heavy phosphorus applications to the C plots were apparently largely ineffective. At least at the time of plant analysis they were not reflected in higher phosphorus reserves than in A. This was presumably due to the repressing action on phosphate absorption of the relatively high nitrate content of the C plants (figs. 4–24), a situation repeatedly illustrated (**1, 2, 10, 11, 12, 13, 15, 23, 30, 33, 36, 42, 50, 51, 57, 60**).

Extensive chemical studies of pineapple plants varying in time of fruiting have not been many. In field experiments extremes of nutrition are not often employed, and where occasionally they have been, low temperatures in many cases have apparently resulted in practically simultaneous blossom-bud differentiation of plants of widely different chemical composition (**37**). Nevertheless, the interrelationships of carbohydrates, nitrate, potassium, and phosphate discussed in the preceding pages seem in harmony with the responses of other species of plants.

That length of night, or low temperature, or both may determine or greatly influence the time of flower-bud differentiation in many plants is well known. It is likewise recognized that under some environmental conditions an excessive supply of available nitrogen in relation to carbohydrates may often lead to limited fruit production, or to greatly delayed fruiting (**25, 42**). While the foregoing points have been emphasized frequently in the literature, the influences of potassium and phosphorus on nitrogenous and carbohydrate reserves and correlated plant responses have been less seldom considered. Such consideration should be given all elements considered essential.

For the reason already noted, heavier phosphorus applications to C than to A (fig. 25) were probably largely ineffective, as they were not reflected in concentrations of phosphorus in the C plants which were any higher than in the A group. But heavy applications of potassium and nitrogen may result in greatly delayed fruiting (fig. 25). This would be expected, for often when potassium is abundant there is relatively free absorption of nitrate, if a liberal supply is available in the soil. Furthermore, these results are in accord with observations by RUSSELL (**42**) that potassium and nitrogen are intimately linked in action. MCCALLA and WOODFORD also reported that limiting the potassium supply of wheat plants resulted in decreased nitrogen content (**32, 33**). HEPLER and KRAYBILL found that high potassium fertilization tended to delay blooming and ripening of tomatoes (**21**). These responses to high potassium nutrition, and many similar cases that might be cited, may be correlated with a comparatively high rate of nitrate absorption and reduction. If this is the case, there necessarily occurs relatively rapid oxidation of carbohydrates or their derivatives (**35, 36**), which under some environmental conditions might lead to deficiency of carbohydrate reserves and the characteristic plant responses noted.

Directly or indirectly, potassium unquestionably accelerates absorption and reduction of nitrate, with concomitant oxidation of nitrogen-free reserves (**38**). But potassium may induce an increase in carbohydrate (no. 1 color) in pineapple plants (figs. 4–24). Therefore the effect of potassium in bringing about more efficient photosynthesis is often to correct the adverse effects on fruit production of

excess nitrogen (42). Thus, particularly in a warm sunny area, potassium could well have the opposite effect on time of fruiting of that shown in figure 25.[1] As always, CO_2 nutrition, which varies with the weather, plays an important role in determining the responses of plants to soil fertilizer treatments, not only in the case of nitrogen (37) but of potassium as well.

That an adequate schedule for phosphate fertilization of pineapple fields cannot be accurately formulated in advance of knowledge of the nitrate requirements of the plants has been indicated (figs. 4–24). When little nitrate is employed, because carbohydrates are low owing to unfavorable weather conditions (37), phosphorus may be absorbed freely—even from soils low in this element (figs. 5, 6). On the other hand, in a year when carbohydrate reserves are high and nitrate is therefore required in abundance, much heavier applications of phosphate are necessary to counteract the suppressing action of nitrate on its intake. The responses of the plants presented in the illustrations of this paper are not aberrant in relation to those of other species. CHAPMAN (10) reports that "nitrogen deficiencies caused inorganic phosphate to accumulate within the oat plant, even in soils deficient in phosphate." MCCALLA and WOODFORD obtained comparable results in wheat (33). Frequently coupled with phosphate accumulation in high concentrations in plants low in nitrate is early senescence of leaves or of the plant as a whole (11, 58, 59).

[1] Although it would seldom be found under conditions of commercial productivity, extreme potassium deficiency, provided some functional leaves are present, sometimes leads to a brief period of carbohydrate accumulation and premature fruiting. This is apparently due, at least in part, to limited use of carbohydrates, in that new protein synthesis from nitrate is practically nil (38, 56).

HEPLER and KRAYBILL (21) found that heavy applications of phosphate hastened maturation of tomatoes, owing to early blooming. RUSSELL (42) emphasized that "phosphatic fertilizers hasten the ripening process." This ripening effect is said to be "well shown on the barley plots at Rothamsted; crops receiving phosphates are golden-yellow in colour while those on phosphate-starved plots are still green." WILLIAMS (58, 59), working with oats, reported that high phosphorus treatment resulted in early maturity, increase in dry matter, and reduction in protein nitrogen content, correlated—at least in part—with the retarding "effect of the high external concentration of phosphate on the intake of nitrogen by the roots."

These responses will not find expression under all environmental conditions (45, 55), as there are many possible limiting factors other than those considered which are often dominant (18, 42, 44, 46, 49). Nevertheless the responses are typical. In harmony with them, MCMURTREY (34) found that phosphorus deficiency delayed maturation of tobacco. Effects of phosphorus deficiency on the delayed ripening of oats and "the protracted and needless intake of nitrate" are reported by STREBEYKO (50). He also found that the higher the amount of phosphate supplied the earlier the plants ceased to take nitrate from the soil and the sooner the grain matured.

Under usual field conditions, it is much more common to find that nitrate limits the intake of phosphate than that the latter suppresses the absorption of nitrate. Even comparatively light applications of nitrogen, in years when weather conditions are not favorable for abundant carbohydrate synthesis, can bring about nitrate accumulation and repression of phosphate absorption in the pine-

apple plant. This is usually accompanied by a low level of carbohydrates and fairly vigorous vegetative growth. It probably is common in other plants. Possibly this situation is comparable with that described by MAYNARD (31). He reports areas where trouble with grazing animals is severe, but "both legumes and grasses grow luxuriantly and apparently provide liberal amounts of nutrient other than phosphorus."

Summary

1. In the pineapple plant a low reserve of nitrate was found adequate for greatest possible yields of fruit when carbohydrates were low. In contrast, when carbohydrates were high maximum production was not obtained unless the plants were essentially filled to capacity with nitrate. In practically the same location a difference of 75 per cent in nitrogen requirements was found in successive years (37).

2. When the concentration of carbohydrates is low and relatively little nitrate therefore supplied, phosphate is freely absorbed, even from soils rather low in phosphorus. In the same site under environmental conditions more favorable for carbohydrate accumulation more nitrate is needed. With higher nitrate there is a proximately corresponding suppression of phosphate absorption, so that it becomes necessary to apply phosphate.

3. The required level of nitrate nutrition, which varies with opportunity for carbohydrate accumulation, is intimately associated with potassium requirements. Under field conditions in Hawaii, nitrate is not absorbed freely by the plant when potassium is low. When carbohydrates are high, and relatively much nitrate therefore required, additional potassium is necessary for nitrate absorption, above that necessary for other functions that potassium performs. Even in the same site the amount of potash required varies with weather conditions from year to year.

4. In plants growing on soils notably low in potassium and phosphorus there was seemingly an interplay of compensating relationships. When potassium was low and absorption of nitrate was in consequence limited, carbohydrate accumulation was also curtailed. In turn, although no phosphate was applied, the low level of nitrate permitted fairly free absorption of phosphate. This apparently resulted in a condition of proximately balanced multiple deficiencies and greatly reduced size of plant and fruit. But, except for size, the plants were much the same in appearance as others with more liberal fertilization, and the fruits were about the same in physical characteristics and in percentage of sugars and total acid as those from plants with a more abundant nutrient supply.

5. Phosphorus is necessary in relatively high concentration just prior to and during blossom-bud differentiation, or severe losses in yields occur. An adequate supply of nitrate in relation to carbohydrates is also essential at that time (37). Application of phosphate to plants high in phosphorus and deficient in nitrate might well bring about still further nitrate deficiency and decrease in yields, although the suppressing action of even high concentrations of phosphate on nitrate absorption is less common than that of nitrate on phosphate intake.

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GROWTH SUBSTANCES AND DORMANCY OF SPORES OF PHYCOMYCES

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(WITH FOUR FIGURES)

Introduction

In earlier papers from this laboratory the low percentage germination of spores of *Phycomyces blakesleeanus* at 25° or 26° C.[1] on an agar medium containing minerals, sugar, asparagine, and thiamine was reported (**4, 5, 6**). The addition to this medium of extracts of various natural products (for example, molasses or extracts of potato tubers) increased germination from 0.5 per cent or less on the basal medium to more than 90 per cent on the medium containing the natural product. Extracts of potato tubers were fractioned by treatment with charcoal. The charcoal eluate (Ca fraction) was somewhat effective in increasing germination, and so was the filtrate (D_R fraction), but the two fractions acting together produced more than an additive effect. Two growth substances were assumed to be present and to account for the effect of the extracts. One of these, factor Z_1, was adsorbed on charcoal; the other, factor Z_2, was present in the filtrate from the charcoal-treated extracts (**6**). Both were thermostable, and neither was identical with any of the known vitamins, amino acids, or other growth substances tested (**5, 6**). Factor Z_1 appeared to be hypoxanthine (**7, 8**). Factor Z_2 remains unidentified (**2, 3, 4**).

It seems, therefore, that the small percentage germination at 25°–26° on a mineral-dextrose medium containing asparagine and thiamine is not because of lack of viable spores of *Phycomyces*, but because on such a medium almost all the spores are dormant. Their dormancy can be broken by the presence of extracts of various natural products.

We have investigated the dormancy of these spores because of interest in the Z factors, which influence not only spore germination but growth and gametic reproduction as well. The problem is of interest also because of the similarity between the effect of the Z factors on *Phycomyces* and that of factors which have been studied by some investigators for other microörganisms (**1, 10**). There is the possibility that the phenomena concerned in the dormancy of the spores may be similar to those determining dormancy in other organisms, including some of the higher plants.

Material and methods

A plus strain of *Phycomyces blakesleeanus* was used throughout the investigation. Unless otherwise stated, stock cultures were grown at 20° in the dark on the basal mineral-dextrose medium containing asparagine, thiamine, and 1.5 per cent Difco agar. Sporangiophores and sporangia were pulled from these cultures with a platinum hook and shaken in distilled water. A loop, 3 mm. in diameter, was used to place a drop of the spore suspension on the surface of 10 ml.

[1] Temperatures throughout this paper are Centigrade.

of the basal medium, to which 1 per cent purified agar had been added, in a petri dish 15 × 90 mm. The dishes were incubated in the dark at 26°–26.5°, and spore counts were made under the low power of a compound microscope after 3–18 hours, depending on the rapidity of germination. As a rule, 100 spores were counted in a single drop. A hand counter helped in recording their numbers. No attempt was made to determine probable errors, as the effects studied were usually sufficiently large and consistent to obviate the necessity of considering them statistically. Most experiments were repeated, many of them several times.

Although our primary interest was in percentage germination, observations were made in many experiments on the growth of the germ tubes and on the diameter and thickness of the mycelial colonies which developed. Some experiments were performed in liquid culture, in which the dry weights of the mycelium were determined.

The basal medium contained per liter: 50 gm. dextrose, 1.5 gm. KH_2PO_4, 0.5 gm. $MgSO_4 \cdot 7H_2O$, 2 gm. asparagine, and 500 μgm. thiamine. To this medium minor elements were added in p.p.m.: 0.005 B, 0.02 Cu, 0.1 Fe, 0.01 Ga, 0.01 Mn, 0.01 Mo, and 0.09 Zn.

All glassware was cleansed with chromic-sulphuric acid cleaning mixture and thoroughly rinsed with tap and distilled water.

Experiments

PERSISTENCE OF DORMANT CONDITION.—At 26° on the basal agar medium, normal untreated spores of *Phycomyces* remained dormant for several days and probably for a month or more. For example, spores from cultures grown 11 days at 20° showed no germination on the basal medium after 24 hours at 26°. On an agar containing potato extract[2] the germination was 98 per cent. The spores on the basal medium showed no germination after 1 week.

CHANGES IN DORMANCY WITH AGE.—Young spores, 1–2 weeks old, showed little or no germination at 26° on the basal medium; a thousand or more spores might be examined before one was found germinating. The percentage germination from progressively older cultures increased to a maximum and then declined. In one experiment spores 1 month old showed 2 per cent germination; this increased to 12 per cent for spores $2\frac{1}{2}$ months old. After 5 months the germination was 4 per cent, and after $8\frac{1}{2}$ months it was less than 1 per cent. A few spores germinated (less than 1 per cent) from cultures 317 days old (table 11).

The lower percentage germination of the younger spores was a dormancy phenomenon, because on agar containing potato extract the germination was nearly 100 per cent. As the spores aged viability decreased. In one experiment spores 4–77 days old germinated 80 per cent or more on the potato-extract agar, but spores 155 days old showed on the same medium but 17 per cent germination, and spores 317 days old showed but 1 per cent (table 11). It seems probable that as the spores grew older changes occurred which broke dormancy; at the same time some spores, probably those ready to germinate, died, until few viable spores—either dormant or active—remained after 317 days. The rapidity of germination changed with age. Spores older than 2 months were slower in germinating than the younger ones.

[2] The potato extract was prepared as described earlier (6). The extract used here contained 500 mg. dry matter per millimeter, and 0.1 ml. was used per petri dish.

Because of the effect of age on germination, spores less than 2 weeks old were customarily used in these experiments.

MINOR FACTORS AFFECTING GERMINATION.—The temperature at which the spores developed had some, though little, effect on the percentage germination. Spores grown at 26° behaved as though they were considerably older than those grown at 20° or lower. Spores from cultures grown 11 days at 26° germinated 3 per cent; spores from cultures of the same age grown at 11° or 20° showed no germination. The tests were carried out at 26° on the basal medium.

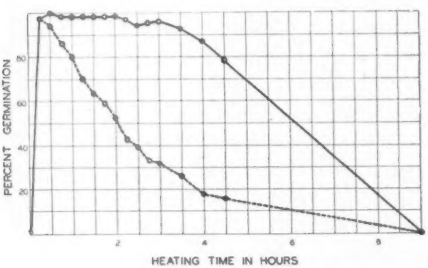


FIG. 1.—Time of heating spores of *Phycomyces* at 50° C. and percentage germination on basal agar at 26° C. Solid line, grown in dark; dotted line, grown in diffuse light.

Spores from cultures grown on the basal medium in the light at 25° for 7 or 14 days germinated a little better than those grown in the dark. The maximum germination observed for 14-day-old spores, grown in faint diffuse light, was 2.3 per cent. However, spores which developed in the light were more easily injured at 50° than were those which developed in the dark. This difference is illustrated by the following experiment. Cultures were grown 8 days in a light-tight box at 25°. By the side of this box other cultures were grown in weak diffuse light. A spore suspension from each set of cultures was then exposed at 50° in a water-bath, and at various times spores were removed and germinated at 26° on the basal medium. The spores grown in the light gave maximum germination after 15 minutes' treatment and progressively lower germination with longer exposures (fig. 1). The spores grown in the dark showed maximum germination with 15 minutes' exposure to heat and little decrease until the treatment exceeded $3\frac{1}{2}$ hours. With 2 hours' exposure at 50° the spores grown in the light germinated 52 per cent; those grown in the dark, 99 per cent.

Light appeared to exert its effect in the early stages of development. Spores grown for 1 week or 10 days in the dark, and then exposed to light for several days, responded as did spores which had been in the dark continuously. There was some indication also that the differences between spores grown in the light and in the dark in susceptibility to heat may disappear as the spores age.

The causes for this difference are obscure. They may be entirely physiological or related in part to the structure of the spores. Spores from cultures grown at 25° in weak diffuse light were smaller, more elongated, and thinner walled; those which developed in the dark were larger, rounder, and thicker walled.

In any event, these observations emphasized the need to consider the light conditions in studies on spore germination of *Phycomyces*, and spores grown at 20° in a dark cabinet were used as standard material. These spores were not in complete darkness. A little light reached them when the cabinet was opened, but this light was insufficient to induce a phototropic response of the sporangiophores.

In contrast to the minor influences of age, light, and the temperature under which the spores had developed, other factors were found to exert a major effect on dormancy.

HYPOXANTHINE AND THE D_R FRACTION.—Both hypoxanthine and the D_R fraction[3] (containing the Z_2 factor) increased the number of spores germinating at 26°. When both factors were present the effect was greater than that produced by the same quantity of either alone. Their action, however, was not entirely specific. There seemed to be something analogous to a mass action effect; that is, a large amount of either hypoxanthine or the D_R fraction (factor Z_2) was effective, and a large amount of the D_R fraction plus a small amount of hypoxanthine had the same effect on germination as a small amount of the fraction and a large amount of hypoxanthine. Although both factors favorably influenced germination and growth, hypoxanthine appeared to be primarily a germination factor, while the D_R fraction had the greater effect on growth. These general statements are illustrated by the following experiment.

Spore suspensions were prepared from cultures grown at 20° which were 8 days and 11 days old, respectively. These suspensions were placed on the basal agar medium and on the same medium to which were added various quantities of hypoxanthine and of a D_R fraction from white potatoes. The spores were germinated at 26°, and colony diameters were measured after 31 hours. In general, the 11-day-old spores showed a greater response to the supplements than those 8 days old. For both lots 100 mμ moles of hypoxanthine[4] were required to affect germination materially. For the older spores 10 mg. of the D_R fraction had some effect, but 100 mg. had more. One mg. of the fraction was effective in combination with 10 or with 100 mμ moles of hypoxanthine; 1 mμ mole of hypoxanthine combined with 10 mg. of the fraction exerted a greater effect than these amounts of either alone. Better growth was obtained with 10 or 100 mg. of the D_R than with 100 mμ moles of hypoxanthine. Best growth occurred when 100 mμ moles of hypoxanthine and 100 mg. of the fraction were present (table 1).

TABLE 1

EFFECT OF VARIOUS AMOUNTS OF HYPOXANTHINE AND D_R FRACTION ON PERCENTAGE GERMINATION OF SPORES OF PHYCOMYCES AND LATER COLONY GROWTH. SPORES 11 DAYS OLD. COLONY DIAMETERS MEASURED AFTER 31 HOURS. TEMPERATURE OF INCUBATION 26° C.

ADDITIONS TO 10 ML. BASAL AGAR MEDIUM	NONE	1 MΜ MOLE HYPOXANTHINE	10 MΜ MOLES HYPOXANTHINE	100 MΜ MOLES HYPOXANTHINE
	Germination (%)			
None	0	0.1	0.1	7
0.1 mg. D_R fraction	0	0	0.1	10
1 mg. D_R fraction	0	0	2	12
10 mg. D_R fraction	7	5	29	48
100 mg. D_R fraction	49	77	98	98
	Colony diameter (mm.)			
None	0*	0*	7‡	8‡
0.1 mg. D_R fraction	0*	0*	6‡	9‡
1 mg. D_R fraction	0*	0*	11‡	10‡
10 mg. D_R fraction	15†	10	15	20
100 mg. D_R fraction	22	24	27	30

* Germination none or almost none. † Thin. ‡ Very thin.

Our observations have led to the assumption that the spores are dormant because of the lack within them of a sufficient amount of Z factors (hypoxanthine

[3] Preparation of Ca and D_R fractions from potato tubers has been described earlier (6).

[4] Somewhat older spores were more sensitive to hypoxanthine. See later section.

and factor Z_2) in available form. It has further been assumed that when a sufficient amount of the Z factors is supplied by addition to the medium of extracts of potato or other natural products, or of hypoxanthine and the D_R fraction, the spores absorb the factors and germination occurs. It would also seem that, at 26°, actively growing mycelium of *Phycomyces* suffers from a partial deficiency[5] for the Z factors, with the result that their addition to the medium increases the amount of growth. This hypothesis will be considered with reference to various factors which break the dormancy of the spores.

TEMPERATURES ABOVE 26° C.—Early in this investigation it was found that relatively short exposures of a spore suspension to a temperature of 50° ± 1° induced a high percentage of germination on the basal agar medium. No germination of untreated spores occurred at this temperature; in fact, the spores were killed if the treatment was sufficiently long, but spores heated a short time at 50° appeared to be activated in such a way that their germination at 26° was increased to nearly 100 per cent (fig. 1).

One procedure in activating spores by heat was as follows: A test tube containing 5 ml. of a spore suspension in distilled water was placed in a hot-air oven heated to 50°. At various intervals loopfuls of the suspension were removed from the tube and placed on the basal medium for germination at 26°. Exposures up to 30 minutes resulted in progressively increased germination, until nearly 100 per cent was obtained with the 30-minute treatment. Exposures longer than 3 hours and 15 minutes resulted in decreased germination. With this procedure the actual temperature to which the spores were subjected was less than 50° for a considerable part of the shorter periods of heating, because of the time required for the 5 ml. of water, initially at room temperature, to reach 50°. When the water was preheated to 50° before the spores were added, 7.5 minutes were sufficient to produce maximum activation.

In many experiments it was found more convenient to immerse the tubes in a waterbath held at 50° than to set them in a hot-air oven. Furthermore, the temperature control in the waterbath was considerably more accurate than that in the hot-air oven. The procedure usually employed for heat activation was to expose the suspensions for between 30 minutes and 1 hour in a waterbath, starting with cold tubes or flasks.

Some differences were noted among various lots of spores in the time at which reduction of germination occurred. In one experiment the time was 3 hours and 15 minutes; in other instances it was as low as 2 hours and 15 minutes. Spores which developed in the light were quickly injured by exposure at 50°, as described earlier. Even when the longer periods of heating did not cause decrease in spore germination, some reduction in growth of the mycelium was observed. However, exposure of between 30 minutes and 1 hour always gave maximum germination and little or no inhibition of growth.

It is reasonable to expect that shorter periods at higher temperatures and longer periods at lower temperatures than those reported would also activate the spores. No extensive investigation of other temperatures was made. It was found, however, that exposure of a suspension for 2 hours and 10 minutes at 35° was ineffective, while 20 hours gave

[5] By a partial deficiency is meant that the organism makes from simple food materials some of the substance but not enough for maximum growth (9).

nearly maximum germination. All the spores were killed when the suspension was heated for 10 minutes at 60° and when 1 ml. of a suspension was placed for 1 minute in a waterbath heated to 80°.

Treatment of dry spores with heat was much less effective than exposure of the spores suspended in water. No effect on germination was observed when open tubes were exposed at 35° for 20 hours. Ten minutes at 100° was partially effective.

HEAT ACTIVATION AND EARLY MYCELIAL GROWTH.—Heat activation not only influenced germination but materially affected the early growth of the mycelium. This is shown by the following experiment.

Spores 10 days old were activated by heating for 2 hours at 50°. The unactivated spores germinated 0.05 per cent and the activated spores 97 per cent. Some flasks containing 25 ml. of the basal solution, and the same solution supplemented with a Ca fraction, a D_R fraction, or both, were inoculated with the activated and others with the unactivated spores. The flasks were incubated at 26° and dry weights determined after 48 and 72 hours. At the end of 48 hours the activated spores had produced nearly as much mycelium in the basal solution as the unactivated spores produced in the presence of the combined Ca and D_R fractions (table 2). After 72 hours the activated spores had formed in the basal solution nearly as much mycelium as the unactivated spores had in a solution supplemented with the D_R fraction, but considerably less than was obtained in the solution containing both Z factors. These results strongly suggest that heat-activated spores contained more available Z factors than unactivated spores. This follows because the activated spores grew in the absence of the Z factors much as the unactivated spores did in their presence. The activated spores did not contain a sufficient amount of the factors to permit maximum growth, however; addition of Z factors to the medium improved their growth as compared with that in the basal solution.

The influence of the D_R fraction and the combined Ca and D_R fractions was in part independent of their effect on germination. This follows because the D_R fraction and the combined Ca and D_R fractions favorably influenced growth of the activated spores, which germinated 97 per cent.

TABLE 2

GROWTH OF UNACTIVATED AND HEAT-ACTIVATED SPORES IN VARIOUS SOLUTIONS

ADDITIONS TO 25 ML. BASAL MEDIUM	DRY WEIGHT (MG.) OF TWO MYCELIA AFTER 48 HOURS IN SOLUTIONS PLUS		DRY WEIGHT (MG.) OF TWO MYCELIA AFTER 72 HOURS IN SOLUTIONS PLUS	
	Unactivated spores	Activated spores	Unactivated spores	Activated spores
None	0.0	8.5	3.6	121.0
0.1 ml. Ca fraction	5.2	9.4	27.2	95.0
0.1 ml. D_R fraction	8.0	25.4	148.0	219.0
Ca fraction and D_R fraction	10.4	26.4	210.0	240.0

PARTIAL ACTIVATION WITH HEAT AND THE Z FACTORS.—As already indicated, heating spores under certain conditions caused maximum germination and increased the growth obtained in the basal solution. These results were interpreted to mean that heating increased the available Z factors in the spores. That heat activation has this effect is supported further by the observation that spores partially activated by heat responded to hypoxanthine and the D_R fraction as

though they contained more of the Z factors than the unactivated spores. For example, in the experiment summarized in table 3, the unactivated spores showed no response to 10 mμ moles of hypoxanthine. The partially activated spores germinated 38 per cent without the hypoxanthine and 95 per cent with it. This could mean that the Z factors in the untreated spores plus that supplied by the hypoxanthine in the medium were insufficient to cause germination; after partial activation the increased Z factors in the spores added to that obtained from the hypoxanthine in the medium allowed 95 per cent germination. The same type of response occurred with the D_R fraction and with the combination of the two Z factors.

TABLE 3

EFFECT OF PARTIAL HEAT ACTIVATION AND Z FACTORS ON PERCENTAGE GERMINATION OF SPORES AND ON COLONY DIAMETERS (MEASURED AFTER 48 HOURS). SPORES 9 DAYS OLD

Additions to 10 ml. basal agar medium	Percentage germination		Colony diameter (mm.)	
	Untreated	Partially activated	Untreated	Partially activated
None	0	38	.....	28
10 mμ moles hypoxanthine	0	95	.....	36
10 mg. D_R fraction	1	95	32	48
Hypoxanthine and D_R fraction	9	98	43	48

If the assumption that partial heat activation increased the amount of available Z factors in the spores is correct, it must be concluded that the quantity of these factors produced by the treatment was insufficient for maximum germination or maximum growth. This follows because the addition of hypoxanthine and the D_R fraction to the medium increased percentage germination and growth (colony diameters) of the activated spores. In this experiment partial activation was accomplished by heating the spore suspension for 5 minutes at 50°. The colony diameters were measured after 48 hours.

PRESENCE OF Z FACTORS IN SPORES.—If heat activation increases the amount of available Z factors in spores, then potentially available Z factors must be present in the spores. Two methods of investigating this question were followed. In one, spores were digested with sulphuric acid and the effects of the digest on germination determined. In the other, the influence of weighed amounts of killed spores on the growth of *Phycomyces* in various solutions was measured.

Spores grown in the basal liquid medium were removed, washed with distilled water, and centrifuged. Spores amounting to 112.5 mg. were digested at boiling temperature with 8N H_2SO_4. The acid was neutralized with $Ba(OH)_2$, the $BaSO_4$ removed, and the filtrate reduced by evaporation to small volume. The digest was added to the basal agar medium. Spores 7 days old showed no germination on this medium; on the same medium plus the digest germination it was 85 per cent; and on potato-extract agar it was 84 per cent.

In a second experiment spores from liquid cultures were removed, washed with distilled water, centrifuged, and activated by heating for 1 hour at 50°. Spores amounting to 8.7, 0.87, and 0.087 mg. were added to flasks containing 25 ml. of the basal solution, to the basal solution plus the Ca fraction (Z_1 factor), and to the basal solution plus the D_R fraction (Z_2 factor). After sterilization the solutions were inoculated with the spores and incubated at 26°. Dry weights

were determined after 72 hours. In the basal medium 8.7 mg. of spores improved growth four times (table 4), and 0.87 mg. had a small favorable effect. The addition of the spores also had a favorable effect in the solutions containing the Ca and the D_R fractions. Apparently a sufficient amount of activated and autoclaved spores favorably affected the early growth of *Phycomyces;* that is, they had the same effect as the addition of hypoxanthine and the D_R fraction to the basal medium.

TABLE 4

PRESENCE OF Z FACTORS IN SPORES. EFFECT OF SPORES ON 72-HOUR GROWTH IN VARIOUS SOLUTIONS

Quantity of spores added to 25 ml. basal medium	Dry weight (mg.) of mycelium produced in two flasks of basal solution supplemented with			
	Nothing	Ca fraction	D_R fraction	Ca and D_R fractions
None	11.7	42.0	139.0	228.0
0.087 mg.	8.5	47.0	190.0	.....
0.87 mg.	15.0	54.0	162.0	.....
8.7 mg.	52.0	87.0	214.0	.....

EXTRACTS FROM HEATED SPORES.—If heat activation transforms Z factors in the spore from an unavailable to an available form, then more Z factors should be extractable from living activated spores than from unactivated ones. This assumption was tested by comparing the effects on the early growth of *Phycomyces* of extracts made with distilled water at 26° from activated and unactivated spores.

Spores were grown in quantity at 20° on the basal solution with asparagine replaced by ammonium glutamate. After 18 days the spores were removed, washed three times with distilled water, and suspended in 20 ml. of distilled water. Ten ml. of the suspension were heated for 20 minutes at 50°. The unactivated spores germinated 23 per cent; the heat-activated spores, 78 per cent. Both suspensions were allowed to stand for 22 hours at 26°. At the end of the extraction period the spores were removed by centrifuging and discarded. Aliquots of each extract were added to 10-ml. quantities of the basal solution, and to the same solution supplemented with hypoxanthine or with the D_R fraction. The extract of 58 mg. of spores was added to each flask. The flasks were sterilized, inoculated, and incubated for 24 hours at 26°. The extract of the unactivated spores improved growth slightly in all

TABLE 5

PRESENCE OF Z FACTORS IN WATER EXTRACTS OF HEAT-ACTIVATED SPORES. GROWTH OF PHYCOMYCES IN VARIOUS SOLUTIONS TO WHICH EXTRACTS OF UNACTIVATED OR HEAT-ACTIVATED SPORES WERE ADDED

Additions to 10 ml. basal medium	Dry weight (mg.) of two mycelia in 48 hours in liquid plus		
	Nothing	Extract unactivated spores	Extract activated spores
	18-day-old spores		
Nothing	0.0	0.9	6.9
50 mμ moles hypoxanthine	20.4	22.0	19.0
13 mg. D_R fraction	38.4	47.0	68.0
Hypoxanthine and D_R fraction	83.0	.....	.....
	9-day-old spores		
Nothing	.....	0.5	3.3
100 mμ moles hypoxanthine	.....	11.8	31.3
13 mg. D_R fraction	.....	31.5	76.8

three solutions, but the extract from the activated spores was markedly effective in the basal solution and in that supplemented with the D_R fraction (table 5).

Since in this experiment the untreated spores showed considerable germination, indicating partial activation somewhere in the process of their preparation, the experiment was in part repeated, using 9-day-old spores. A suspension of 168 mg. of spores in 25 ml. of water was heated for 1 hour at 50°. An equal weight of untreated spores was suspended in 25 ml. of distilled water. The unactivated spores germinated 0.5 per cent; the activated, 96 per cent. Both suspensions were allowed to stand for 24 hours at 26°. The spores were then centrifuged off and discarded and the extracts evaporated to 12 ml. Aliquots of each extract were added to 10-ml. quantities of the basal solution and to the same solution plus hypoxanthine or the D_R fraction. Each flask received the extract of 28 mg. of spores. After sterilization the flasks were inoculated and incubated at 26° for 48 hours. The extracts from the activated spores had a distinctly greater effect on the early growth of *Phycomyces* than had those from the unactivated spores (table 5).

LOSS OF VITALITY OF HEAT-ACTIVATED SPORES.—If the Z factors diffuse into distilled water more rapidly from heat-activated spores than from unactivated, heat-activated spores might be expected to lose their vitality more rapidly when suspended in distilled water than those not so activated. This assumption was confirmed by the following experiment.

A suspension of spores 16 days old was made in 10 ml. of distilled water. Half the suspension was heated for 1 hour at 50°. Both suspensions were placed at 26° and germination determined on potato-extract agar at weekly intervals. At the end of 5 days the unactivated spores germinated 98 per cent; the activated, 88 per cent. After 18 days the unactivated spores germinated 77 per cent; the activated, 10 per cent. After 32 days the unactivated spores germinated 76 per cent; the activated, 1 per cent.

EFFECT OF TEMPERATURES BELOW 26°.—Not only may dormancy be broken by suitable treatment with temperatures above 26°, but the spores respond also to short exposures below 26° under certain conditions, as illustrated by the following experiment.

TABLE 6

TWO-HOUR EXPOSURE ON BASAL AGAR MEDIUM AT TEMPERATURES INDICATED AND PERCENTAGE GERMINATION AT 26° C.

EXPOSURE ON BASAL AGAR MEDIUM (°C.)	PERCENTAGE GERMINATION OF SPORES			
	7 days	10 days	14 days	17 days
3	0.5	8	0.9	5
11	15	10	7.5	12
15	10	19	5	16
20	5	4	0.5	1
26	0	0.1	0.1	0.1
35	.....	0	0.1	0.1

Spores grown at 20° in the dark were placed on the basal agar medium. The petri dishes were exposed for 2 hours at 3°, 11°, 15°, 20°, 26°, or 35° and then transferred to 26° for germination. Spores were treated in this fashion when they were 7, 10, 14, and 17 days old. The spores exposed at 26° and at 35° for 2 hours germinated 0–0.1 per cent; at 20°, 0.5–5 per cent; at 15°, 5.0–19.0 per cent; at 11°, 7.5–15 per cent; and at 3°, 0.5–8.0 per cent (table 6). It is probable that some of the variations in the effect of a given temperature on spores of different ages was because the petri dishes and agar were not precooled but were at

room temperature before the 2-hour treatment.

At any rate, numerous experiments showed that 2 hours' exposure of spores at 11° or 15° on the basal agar medium broke dormancy to a considerable extent;[6] 20° was less effective; and 3° had little effect. Had the plates been precooled, 3° would perhaps have had no effect. Exposures at 26° and 35° were ineffective (table 6).

There was one noteworthy difference between the action at 50° and of temperatures below 26°. Spores suspended in distilled water were activated at 50° but not by the lower temperatures, which were effective only when the spores were exposed on the basal agar medium or in the basal nutrient solution. The ineffectiveness of exposure at 3° as compared with that at 11° or 15° suggests that the action of the favorable lower temperature is associated in some way with metabolism of the spore. This assumption is supported by the fact that the lower temperatures were not effective unless the components of a full nutrient solution were available during the exposure.

If the presence of sufficient amounts of available Z factors is necessary for germination, as assumed, then it must also be assumed that metabolism of the spores for 2 hours at 11° or 15° produces more available Z factors than at 26°. Since the rate of metabolism is doubtless more rapid at 26° than at 11°, apparently the kind of metabolism at 11° or 15° differs from that at 26° and results in more rapid accumulation of available Z factors.

The temperature at which spores were grown seemed to have little effect on the percentage germination on the basal agar medium, although there was considerable difference in the response to the Z factors and to short exposure to low temperature.

Spores 11 days old, grown at 11°, 20°, and 26°, were germinated on the basal agar medium and on the same medium supplemented with the Z factors (table 7). Petri dishes containing spores grown at each of these temperatures were exposed for 2 hours at 11° and then placed at 26° for germination. In general, the response of the spores to the Z factors and to exposure to low temperature decreased with the temperature at which they were grown. Spores grown at 11° showed the greatest response to the Z factors; those grown at 26° benefited less from short exposure to 11° than those grown at 11°. Spores grown at 11° appeared to contain a greater quantity of the Z factors which could be made available by exposure at 11°.

The response to hypoxanthine of the 20° and 26° spores exposed 2 hours at 11° was great, suggesting that much of the

TABLE 7

EFFECT OF SHORT EXPOSURE AT 11° C. ON SPORES GROWN AT VARIOUS TEMPERATURES. SPORES 11 DAYS OLD

Additions to 10 ml. basal agar medium	Percentage germination at 26° C.					
	Of spores grown at			After 2 hours at 11° C. of spores grown at		
	11°	20°	26°	11°	20°	26°
None	9	0	3	93	39	17
100 mμ moles hypoxanthine	41	7	16	96	77	62
100 mg. D_R fraction	84	49	24	94	89	90
100 mμ moles hypoxanthine and D_R fraction	98	98	89	98	99	86

[6] Exposure of 2½ hours at 11° on precooled agar media gave 50 per cent germination when the check showed 0.1 per cent.

Z_2 factor had accumulated at the lower temperature.

ACETATE.—The action of these various physical factors can be duplicated in part by chemicals, which also may be assumed to affect the availability of Z factors. The addition of 10 mg. acetate (prepared from acetic acid redistilled from glass) as sodium acetate per petri dish containing 10 ml. of the basal medium produced maximum germination; 1 mg. was partially effective.

Spores germinated also after soaking in the basal nutrient solution containing acetate. They were suspended for 3 hours at 26° in 10 ml. of the basal solution containing 10 mg. acetate as sodium acetate and then placed on the basal agar medium. Swollen spores were observed within 30 minutes, and maximum germination occurred; but treatment in distilled water containing acetate for 2–26 hours was ineffective. An attempt was made to define the portion of the nutrient solution concerned in promoting the acetate effect. The action of acetate in a solution of the minerals of the basal solution and thiamine was slight (8 per cent germination); it was somewhat greater in a solution of dextrose, asparagine, and thiamine (40 per cent); with the complete nutrient solution it was 99 per cent. Apparently the activity of acetate in breaking dormancy was associated in some way with the presence of the constituents of a complete nutrient solution.

EFFECT OF OTHER ORGANIC ACIDS.—The effect of the sodium salts of other organic acids[7] also was investigated. One mg. of each acid in the form of its sodium salt was added to a petri dish containing 10 ml. of the basal agar medium. This was repeated, using 10 mg. of each acid per petri dish. The acids fell in the following order: propionate > acetate > pyruvate > *n*-butyrate > *n*-valerate > *iso*-butyrate, formate. The propionate at 10 mg. gave maximum germination but reduced growth materially; at 1 mg. it was superior to acetate in its effect on germination and had little or no effect on growth. The pyruvate at 10 mg. materially increased germination but also completely inhibited growth; at 1 mg. it had no effect on germination. The *n*-butyrate at 10 mg. increased germination but was ineffective at 1 mg. The *n*-valerate at 10 mg. inhibited germination but was slightly active at 1 mg. *Iso*-butyrate and formate were slightly effective at 10 mg. and ineffective at 1 mg. The citrate, diethylacetate, fumarate, lactate, malate, malonate, oxalate, succinate, and tartrate were ineffective. The *n*-caproate at 10 mg. completely inhibited germination and was ineffective at 1 mg.

RELATION BETWEEN ACETATE AND Z FACTORS.—If acetate breaks dormancy of the spores by releasing Z factors already present but in unavailable form, the effects of acetate and of the Z factors should be expected to supplement each other. This appeared to be true. The germination of spores partially activated by acetate was improved by the presence of hypoxanthine or the D_R fraction. In one experiment spores 13 and 35 days old were placed on the agars indicated (table 8). In general, the older spores gave the greater responses. On the basal agar medium no germination of the spores 13 days old occurred. The addition of 1 mg. acetate increased germination to 13 per cent. Hypoxanthine and 1 mg. acetate gave 71 per cent, although germination with hypoxanthine alone was 7 per cent. Germination with the D_R fraction alone was 9 per cent, and with

[7] With the exception of the acetate, the acids used were obtained from Eastman Kodak Company and were not especially purified.

the D_R fraction and 1 mg. acetate it was 76 per cent. Maximum germination was obtained on the basal medium to which 10 mg. acetate were added. The Z factors affected the mycelial growth as measured by colony diameters, even in the presence of 10 mg. acetate, where complete germination occurred throughout. Since 10 mg. acetate approaches the maximum which may be used without reducing growth, it would appear that acetate was not a complete substitute for the Z factors.

TABLE 8

EFFECT OF ACETATE AND Z FACTORS ON GERMINATION AND COLONY DIAMETERS OF PHYCOMYCES. SPORES 13 DAYS OLD

Addition to 10 ml. basal agar medium	Percentage germination of spores on basal medium plus			Colony diameters (mm.) after 28 hours on basal medium plus		
	Nothing	1 mg. acetate	10 mg. acetate	Nothing	1 mg. acetate	10 mg. acetate
None	0	13	97	.....	5	12
10 mμ moles hypoxanthine	7	71	97	9	12	15
13 mg. D_R fraction	9	76	97	18	19	20
Hypoxanthine and D_R fraction	64	99	99	23	23	23
0.1 ml. potato extract	93	.....	.....	30	.....	.....

PYRIDINE.—Spores soaked in 5 per cent aqueous pyridine for 70 hours at about 3° gave high percentage germination. Treatment for 24 hours at 26° with 5 per cent pyridine killed most of the spores; 2.5 per cent gave 74 per cent germination; 1.25 per cent gave 12 per cent germination; 0.625 per cent gave 2 per cent; and water gave less than 0.5 per cent. The effect of the pyridine increased with the time of treatment. For spores which showed less than 0.5 per cent germination, 2 hours at 26° with 2.5 per cent pyridine gave 7 per cent germination; 6 hours, 24 per cent.

RELATION OF PYRIDINE, ACETATE, AND THE Z FACTORS.—The combined action of pyridine and acetate seemed to be similar to that of the Z factors. Spores 16 days old were suspended in water and in 0.625 and 1.25 per cent pyridine for 24 hours at 26°. They were then placed on the basal medium and on the same medium plus acetate and the Z factors. On the basal medium (table 9) germination was 0.5 per cent; acetate increased it to 38 per cent; acetate and 0.625 per cent pyridine to 73 per cent; and acetate and 1.25 per cent pyridine to 97 per cent. Germination on the agar containing hy-

TABLE 9

EFFECT OF PYRIDINE, ACETATE, AND Z FACTORS ON GERMINATION OF 16-DAY-OLD SPORES OF PHYCOMYCES

Addition to 10 ml. basal agar medium	Percentage germination after 24 hours					
	In water		In 0.625 pyridine		In 1.25 pyridine	
	No acetate	1 mg. acetate	No acetate	1 mg. acetate	No acetate	1 mg. acetate
None	0.5	38	2	73	12	97
10 mμ moles hypoxanthine	6	88	18	96	49	99
13 mg. D_R fraction	5	80	30	92	65	99
10 mμ moles hypoxanthine and 13 mg. D_R	60	94	74	99	97	99
100 mμ moles hypoxanthine	12	.....	27	.....	68	.....
100 mμ moles hypoxanthine and 13 mg. D_R	47	.....	67	.....	94	.....

poxanthine was 6 per cent; hypoxanthine and acetate, 88 per cent; and for spores treated with 0.625 per cent pyridine and germinated on the agar containing both hypoxanthine and acetate it was 96 per cent. However, growth (as measured by colony diameters) of those spores treated with 1.25 per cent pyridine and grown on

TABLE 10

EFFECT OF PYRIDINE, ACETATE, AND Z FACTORS ON COLONY DIAMETERS (CF. TABLE 9)

ADDITION TO 10 ML. BASAL AGAR MEDIUM	COLONY DIAMETER AFTER 24 HOURS					
	In water		In 0.625 pyridine		In 1.25 pyridine	
	No acetate	1 mg. acetate	No acetate	1 mg. acetate	No acetate	1 mg. acetate
None	6*	11	6*	9	6*	8
10 mμ moles hypoxanthine	13*	12	12	13	14	11
13 mg. D_R fraction	18	24	19	23	19	22
10 mμ moles hypoxanthine and 13 mg. D_R	25	26	22	24	24	24
100 mμ moles hypoxanthine	24	.....	14	.....	12	.....
100 mμ moles hypoxanthine and 13 mg. D_R	24	.....	23	.....	20	.....

* Very thin.

the acetate agar was favorably influenced by the Z factors (table 10). This effect was independent of germination, since these spores germinated completely on all media. This suggests that although pyridine and acetate have the same kind of effect on the spores as the Z factors, their effect is not great enough to produce maximum growth. This is to be expected. The quantity of Z factors released in a single spore must of necessity be small. If the factors are used in growth, the quantity in a single spore must be quickly exhausted.

WATER.—Spores showed relatively little change in dormancy for some months if left in the sporangia. However, 2 weeks' immersion in distilled water at 26° broke the dormancy for a considerable percentage of them, and 4 weeks was still more effective. This is illustrated by the following experiment.

Spores grown at 20° for 8, 30, 78, 113, and 264 days were suspended in distilled water and the tubes set in an incubator at 26°. Spores were transferred to the basal agar medium and to potato-extract agar when the experiment was initiated and after 14 and 28 days. On the basal medium the 8-day spores showed no germination at the start, 48 per cent after 14 days, and 81 per cent after 28 days. On the potato-extract agar germination was 96, 99, and 93 per cent. Germination of the older spores also was increased by soaking but was less after 28 days than after 14 days.

AGE, ACETATE, AND THE Z FACTORS.—Spores of various ages were germinated on the basal agar medium, and on the same medium to which 1 mg. acetate, 10 mg. acetate, 10 mμ moles hypoxanthine, 10 mg. D_R fraction, or 0.1 ml. extract of potato tubers was added separately and in combination.

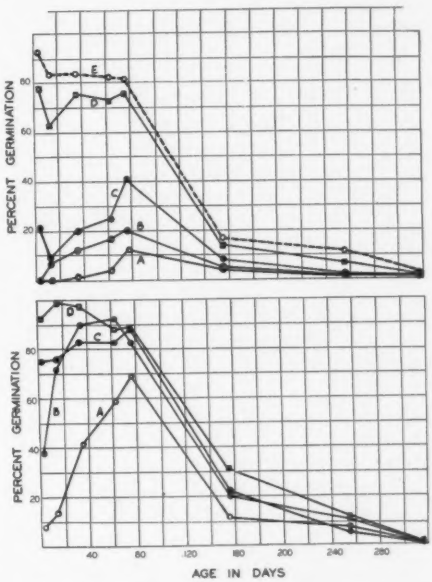
Germination on the potato-extract agar and on the acetate agar showed that the percentage of viable spores decreased with age. The decrease was relatively small up to 77 days and rapid between 77 and 155 days (table 11). The percentage of dormant spores decreased as the spores grew older; the apparent decrease in the proportion of dormant spores between 77 and 255 days was due to the decrease in the percentage of viable spores. The proportion of dormant spores stimulated to

germinate by hypoxanthine or the D_R fraction increased with age. There appeared to be a slow accumulation of available Z factors in the spores as they grew older, with the result that larger and larger proportions of them germinated on the basal agar medium and were induced to germinate by the presence of one of the Z factors in the agar (table 11; fig. 2). By supplying both the Z factors in the medium it was possible to induce a greater proportion of the spores to germinate than could be accomplished by furnishing only one. This is interpreted to mean that both factors slowly became available, but at any time the amount present in many spores was below the threshold necessary for germination and required supplementing through a supply in the medium. Comparison of the germination in the presence of acetate and that in the presence of the two Z factors, or of potato extract,

TABLE 11

RELATION OF AGE, ACETATE, AND Z FACTORS TO GERMINATION OF SPORES OF PHYCOMYCES AT 26° C.

Age of spores (days)	Percentage germination on basal agar medium plus: Nothing	Hypoxanthine	D_R fraction	Hypoxanthine and D_R fraction	Potato extract
4	0	0	22	78	93
13	0	7	9	64	84
35	2	11	20	76	84
61	4	17	25	73	82
77	12	20	41	76	81
155	4	5	8	14	17
255	Less than 1	4	4	7	11
317	Less than 1	Less than 1	Less than 1	Less than 1	1
	Plus 1 mg. acetate				
4	7	38	75	93	.....
13	13	71	76	99	.....
35	41	90	83	97	.....
61	59	93	83	88	.....
77	69	83	88	89	.....
155	12	20	22	31	.....
255	8	11	6	13	.....
317	Less than 1	Less than 1	Less than 1	Less than 1	.....
	Plus 10 mg. acetate				
4	93	97	95	95	.....
13	97	97	97	99	.....
35	99	98	97	99	.....
61	94	93	96	94	.....
77	86	91	85	87	.....
155	24	15	20	24	.....
255	10	10	10	11	.....
317	Less than 1	1	Less than 1	1	.....



FIGS. 2, 3.—Fig. 2 (above), percentage germination of spores of various ages on various media. *A*, on basal medium; *B*, plus hypoxanthine; *C*, plus D_R fraction; *D*, plus hypoxanthine and D_R fraction; *E*, on potato-extract agar. Fig. 3 (below), percentage germination of spores of various ages on various media, all containing 1 mg. acetate as sodium acetate per petri plate with 10 ml. medium. *A*, basal medium; *B*, plus hypoxanthine; *C*, plus D_R fraction; *D*, plus hypoxanthine and D_R fraction.

shows that insufficient amounts of these materials were used to produce a maximum effect. The effect of 1 mg. acetate increased with the age of the spores (table 11; fig. 3). Its action on young spores was more effectively supplemented with the D_R fraction than with hypoxanthine, but for spores 35 and 61 days old hypoxanthine was more effective than the D_R fraction. One mg. acetate affected the spores as would be expected if the Z factors were deficient in the young spores and increased as they aged. Germination on the agar containing 10 mg. acetate was maximum throughout.

TABLE 12

EFFECT OF AGE, ACETATE, AND Z FACTORS ON COLONY DIAMETERS (CF. TABLE 11)

Age of spores (days)	Diameter of colonies (mm.) after 28 hours on basal agar medium plus				
	Nothing	Hypoxanthine	D_R fraction	Hypoxanthine and D_R fraction	Potato extract
4	0	0	15	20	32
13	0	9	18	23	30
35	4	11	20	25	28
61	6	12	20	22	26
77	6	11	20	19	24
155	6	6	18	17	21
255	0	0	10	14	18
317	0	0	6	6	10
	Plus 1 mg. acetate				
4	6	10	22	22	.....
13	5	12	19	23	.....
35	10	16	22	24	.....
61	11	12	21	22	.....
77	7	13	19	20	.....
155	7	9	13	18	.....
255	5	5	12	13	.....
317	0	0	0	0	.....
	Plus 10 mg. acetate				
4	14	16	22	23	.....
13	12	15	20	23	.....
35	12	14	23	23	.....
61	11	14	17	22	.....
77	11	12	15	18	.....
155	8	9	14	15	.....
255	5	6	10	12	.....
317	0	0	3	4	.....

Although acetate had a marked effect on germination, its effect on colony growth was not so great as that of the Z factors. For example, the maximum growth in the basal agar plus 10 mg. acetate was 14 mm. (for the younger spores), while on the agar containing hypoxanthine and the D_R fraction it was 20 mm. for the same spores, and 25 mm. for spores 35 days old (table 12). In other words, although acetate markedly affected germination, its effect on growth was slight. This is evident (fig. 4) if the colonies produced from the spores 4, 13, and 35 days old on the agar containing 10 mg. acetate (9) are compared with those on the agar containing the two Z factors (4).

Discussion

It is assumed that the active factors concerned in breaking the dormancy of spores of *Phycomyces* and in improving its early mycelial growth in a basal mineral-dextrose medium containing thiamine and asparagine at 26° were hypoxanthine (factor Z_1) and factor Z_2, or physiologically equivalent substances. The action of other effective agents, including heat, low temperature, organic acids, pyridine, and soaking in water, is interpreted on the assumption that these treatments increased the quantity of Z factors available in the spores.

Although the final result seemed to be much the same, there was some difference in the conditions under which these various physical and chemical agents were effective. High temperature, pyridine, and soaking in water at 26° seemed

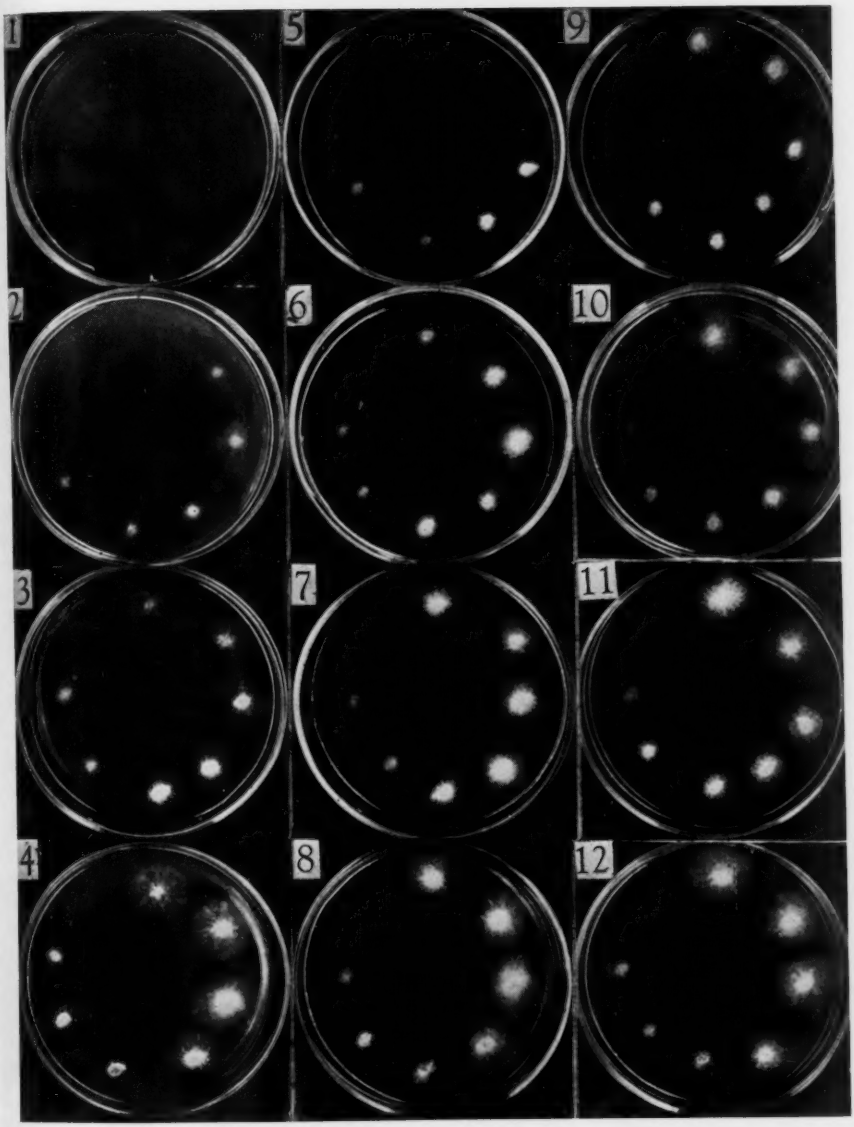


FIG. 4.—Colony growth of spores of *Phycomyces* of various ages on various media after 28 hours at 26° C. Age of spores in days on each plate beginning at top and proceeding clockwise: 4, 13, 35, 61, 77, 155, 255, and 317. Media as follows: *1*, *5*, *9*, basal medium; *2*, *6*, *10*, plus hypoxanthine; *3*, *7*, *11*, plus D_R fraction; *4*, *8*, *12*, plus hypoxanthine and D_R; *5*, *6*, *7*, *8*, plus 1 mg. acetate; *9*, *10*, *11*, *12*, plus 10 mg. acetate.

to set Z factors free, independent of the metabolic activity of the spore. On the other hand, low temperature and acetate appeared to act by inducing a type of metabolic activity in the spore which increased the quantity of available Z factors. Evidence for the validity of this hypothesis as an explanation for the effect of heat-activation may be summarized as follows:

Spores were demonstrated to contain material which affected germination and growth as the Z factors do.

The germination of partially heat-activated spores and the early mycelial growth of spores fully activated by heat agreed with the assumption that the quantity of these factors in the spores had been increased by the heating.

Material which affected early mycelial growth, as the Z factors do, diffused in greater quantity from heat-activated spores than from unactivated ones.

The evidence for a similar action by the other factors (acetate, pyridine, low temperature, and soaking in water) is not so complete. It is based chiefly on the fact that spores partially or completely activated by these agencies responded in media containing hypoxanthine or the D_R fraction as though the amount of the Z factors in them had been increased by the treatment concerned.

Although the hypothesis proposed is the most satisfactory devised so far, there are several questions in relation to it which need discussion.

Why are the Z factors not more specific in their effects on germination? A sufficient amount of either breaks dormancy. Several answers to this question may be suggested, none of which is entirely satisfactory.

The spore population may be mixed; that is, some spores contain sufficient available hypoxanthine but are not capable of germinating because of a lack of the Z_2 factor; others contain sufficient Z_2 factor but not enough hypoxanthine; a few contain enough of both and germinate on the basal medium.

There is also the possibility that there may be a kind of mass relation between these two factors, such that the product of their masses, within certain limits, induces germination and favors early mycelial growth.

A third possibility is that the D_R fraction, used here as a source of the Z_2 factor and which is a mixture of many substances, may be effective because it contains one of the active chemical agents; for example, organic acids. This interpretation is doubtful. The D_R fraction is active in amounts so small that if its activity were caused by any of the organic acids tested those acids would constitute 10 per cent or more of the fraction. This is not probable, as no migration of the active material to the anode chamber was observed when a D_R fraction was electrodialyzed. Furthermore, it has not been possible to duplicate the effect of the fraction on growth by any of the organic acids tested.

What evidence is there for assuming that the various chemical and physical agencies act through the intermediary of the Z factors and not direct? The most important evidence is that none of the treatments (heat, acetate, pyridine, etc.) was as effective as the addition of hypoxanthine and factor Z_2 (in the D_R fraction) to the medium. This was not true for germination but applied to early mycelial growth. For example, although acetate produced maximum germination it had little effect upon growth and did not replace the D_R fraction in this respect. The growth of heat-activated spores was

improved by the addition of the Z factors to the medium. Similar considerations may be applied to the other treatments. If one of the other factors was the primary agent, then it should be possible to duplicate by its use the effects of the Z factors. On the other hand, if the other factors act through the Z factors as intermediaries, spore treatments would be expected to produce less than enough to give maximum effects on later growth. This would be because the quantity of Z factors released in a spore as the result of any specific treatment must of necessity be minute and insufficient to produce maximum effect on later growth, even though the quantity was adequate to induce germination.

Of other explanations which might be suggested, change in the permeability of the spore membranes seems most probable. It might be assumed that the Z factors, heat, low temperature, organic acids, and pyridine affect germination by increasing the permeability of the membranes. However, this would not account for effects noted on later growth.

In fact, the hypothesis which assumes the Z factors as the primary agents concerned appears to fit the results better than any other. A final decision on its validity will probably not be possible until the Z_2 factor has been identified. Discussion of the application to other examples of dormancy of these results and their explanation would not be profitable at this time. The similarity between the treatments found effective in breaking the dormancy of spores of *Phycomyces* and those used for other dormant organs or entire plants is obvious. It seems reasonable to assume that dormancy in some instances results from a temporary deficiency of one or more essential metabolites. Time or a suitable treatment supplies or induces the formation of the deficient and essential substances, and breaks or shortens the dormant period.

Summary

Spores of *Phycomyces* germinate poorly at 26° C. on a mineral-dextrose agar medium containing thiamine. The percentage of germination increases somewhat with age of the spores, reaching a maximum of about 12 per cent after $2\frac{1}{2}$ months. It is affected slightly by the temperature at which the spores are grown and by their exposure to light during development. The addition to the medium of extracts of potato tubers or other natural products, of hypoxanthine and a D_R fraction (Z factors), or of acetate and some other organic acids increases germination to nearly 100 per cent. Treatment of spores with aqueous pyridine, 2 hours' exposure to temperatures of 11° or 15°, or treatment for 30 minutes to 1 hour at 50°, has the same favorable effect. Failure of the spores to germinate on the basal medium is interpreted as a dormancy phenomenon. The dormant spores are considered to lack sufficient available Z factors for germination. The extracts of natural products or the Z factors furnished in the medium supply the deficiency, which may also be met by treatment with heat, cold, acetate, or pyridine. These treatments are thought to change the Z factors in the spores from an unavailable to an available form.

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HISTOLOGICAL RESPONSES OF VICIA FABA TO INDOLEACETIC ACID

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 548

BARBARA F. PALSER

(WITH TWENTY-THREE FIGURES)

Introduction

The gross and histological responses of several varieties of plants to various growth-regulating substances, applied laterally to intact and apically to decapitated stems, have been reported. A few recent papers (**8, 10, 19**) suggest that certain growth substances may induce polyploidy in nuclei of the treated organs. There is no evidence recorded for bean (**17**), *Iresine* (**14**), tomato (**6**), or *Lilium* (1) that indoleacetic acid does induce polyploidy. On the contrary, BEAL found evidence in *Lilium* indicating that all stages of mitosis were regular and chromosome structure normal throughout division.

For the present investigation, plants of *Vicia faba* were grown in pots containing garden soil under ordinary greenhouse conditions during the autumn, winter, and spring of 1941–1942. Following germination, the cotyledons and the first or cotyledonary node of *Vicia* remain underground; small bracts are present at the second and third nodes, and the first true leaf with its large stipules is at the fourth node. Stems were decapitated at the upper end of the fourth internode, just below the fifth node, at the time the second true leaf was beginning to expand and the fifth internode beginning to elongate slightly (fig. 1*A*). The cut surface was covered with a layer of a 1.5 per cent mixture of indoleacetic acid in lanolin or with lanolin alone.

The tips of the treated plants were collected at hourly intervals from 15 to 48 hours following treatment, and after that at 4-hour intervals up to 104 hours. Others were allowed to grow for a month or more before collection. The tips were killed and fixed in Navashin's solution, and imbedded according to the butyl alcohol-paraffin method. Sections were cut at 10 μ and stained in a modified triple stain.

Plants of a series grown in late spring and early summer, 1942, and treated as before, were collected at intervals, sectioned freehand, and stained with iodine-potassium iodide solution. These sections showed the effects of treatment on starch stored in the endodermis and on the chloroplasts present in the outer cortex.

Observations

GROSS RESPONSES

During the first 24 hours after treatment little or no change could be observed. Occasionally, if the tissue when treated was in a younger, more immature stage than usual, a very slight swelling could be seen. During the next 24 hours the swelling of the tip, although never very great, became distinct. The bright green of the stem became paler in the swollen region. The amount of swelling increases with length of time after treatment (fig. 1*C–F*). The plants treated with lanolin alone showed no similar

swelling or decrease in color (fig. 1*B*), and on them lateral shoots developed rapidly at the second and third nodes. Shoots also developed on plants treated with indoleacetic acid but were slower in starting and somewhat slower in later development. The tumors that had developed for a month or more showed a slightly darkened, roughened surface, and some showed a small amount of callus proliferation. Plants decapitated and left untreated showed a dry exposed surface but otherwise little change over a considerable period of time. The relative size of tumors formed on stems of *V. faba* following treatment was much smaller than those formed on bean in a corresponding length of time. A young tumor may extend 2–3 mm. back from the cut surface and the fading of color slightly farther. Old tumors do not extend much farther back in the stems than young ones, the longest extending 3–5 mm. The increase in diameter varies from an amount barely visible to once and a half or twice the original diameter.

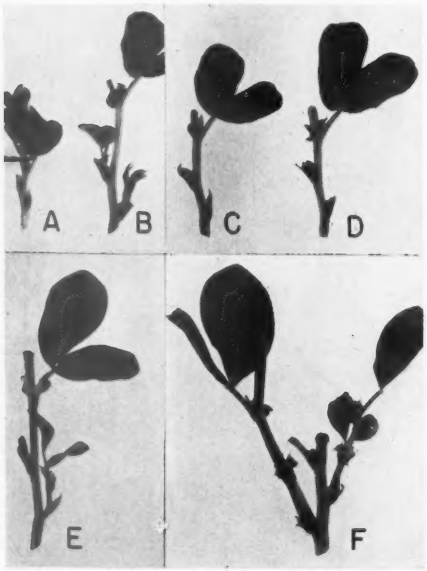
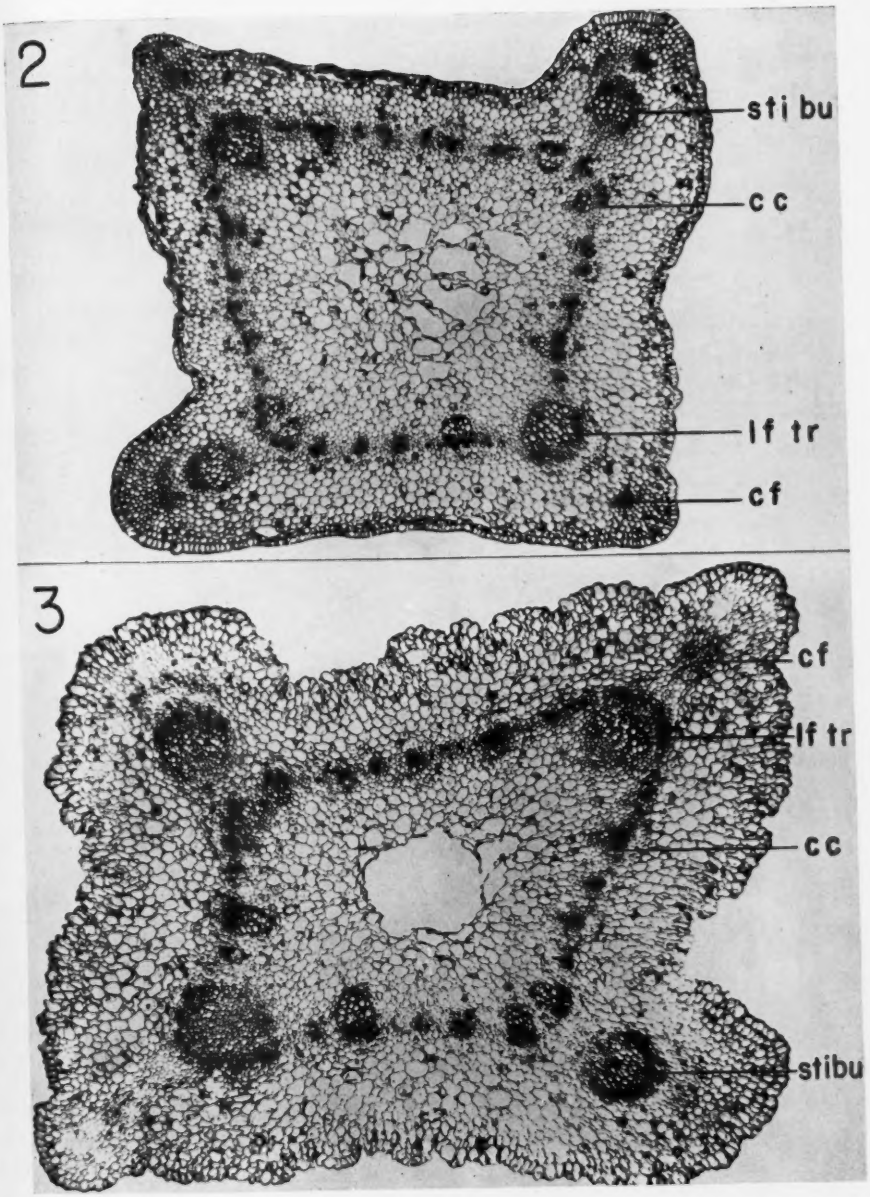


FIG. 1.—*A*, *V. faba* at time of treatment, showing region of decapitation. *B*, decapitated plant treated with lanolin alone, 94 hours after treatment. *C–F*, decapitated plants treated with 1.5 per cent indoleacetic acid in lanolin (*C*, 30 hours after treatment; *D*, 71 hours; *E*, 94 hours; *F*, 30 days after treatment).

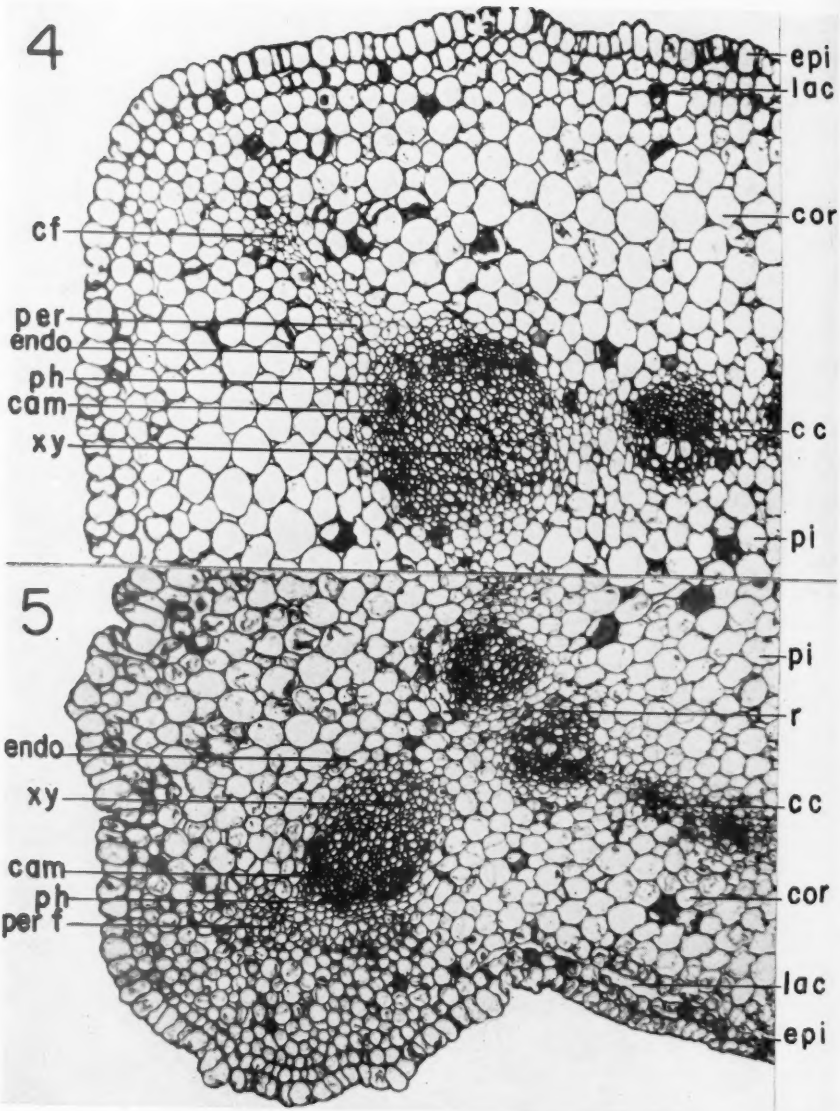
HISTOLOGICAL DETAILS

The transition region in *V. faba* extends through the first two internodes and is in many respects similar to that in pea (**7, 24, 15**). Typical stem anatomy is present in the treated fourth internode. At the time of treatment many of the cells are still immature, but the regions are differentiated and can be easily distinguished. Figure 2 shows a cross-section of the fourth internode 32 hours after treatment with lanolin alone. A central cylinder of vascular bundles is evident, with two polar bundles much more prominent than the others, the leaf traces for the leaves at the fifth and sixth nodes. In the cortex outside each trace is a small bundle of fibers which departs into the leaf with the leaf trace. Such a leaf trace and cortical bundle are shown in figure 4. The leaf gap is closed by the adjacent bundles of the central cylinder. Large fibrovascular bundles are also present in the cortex in the corners alternate with those in which the leaf traces are situated. These are the stipular bundles (fig. 5). At the fifth node each stipular bundle divides into two, one-half supplying the stipule and the other half remaining in the cortex through the fifth internode. The bundles of the central cylinder vary in size and are separated by parenchymatous rays of differing widths. Rays one cell in width are formed in the secondary xylem.

Surrounding the vascular cylinder and the stipular bundles is an endodermis,



FIGS. 2, 3.—Cross-sections of fourth internode of *V. faba* just below fifth node. Fig. 2, 0.85 mm. from cut surface; 32 hours after treatment with lanolin alone. Fig. 3, 0.85 mm. from surface; 31 hours after treatment with 1.5 per cent indoleacetic acid in lanolin. *sti bu*, stipular bundle; *lf tr*, leaf trace; *cf*, cortical strand of fibers; *cc*, central cylinder.



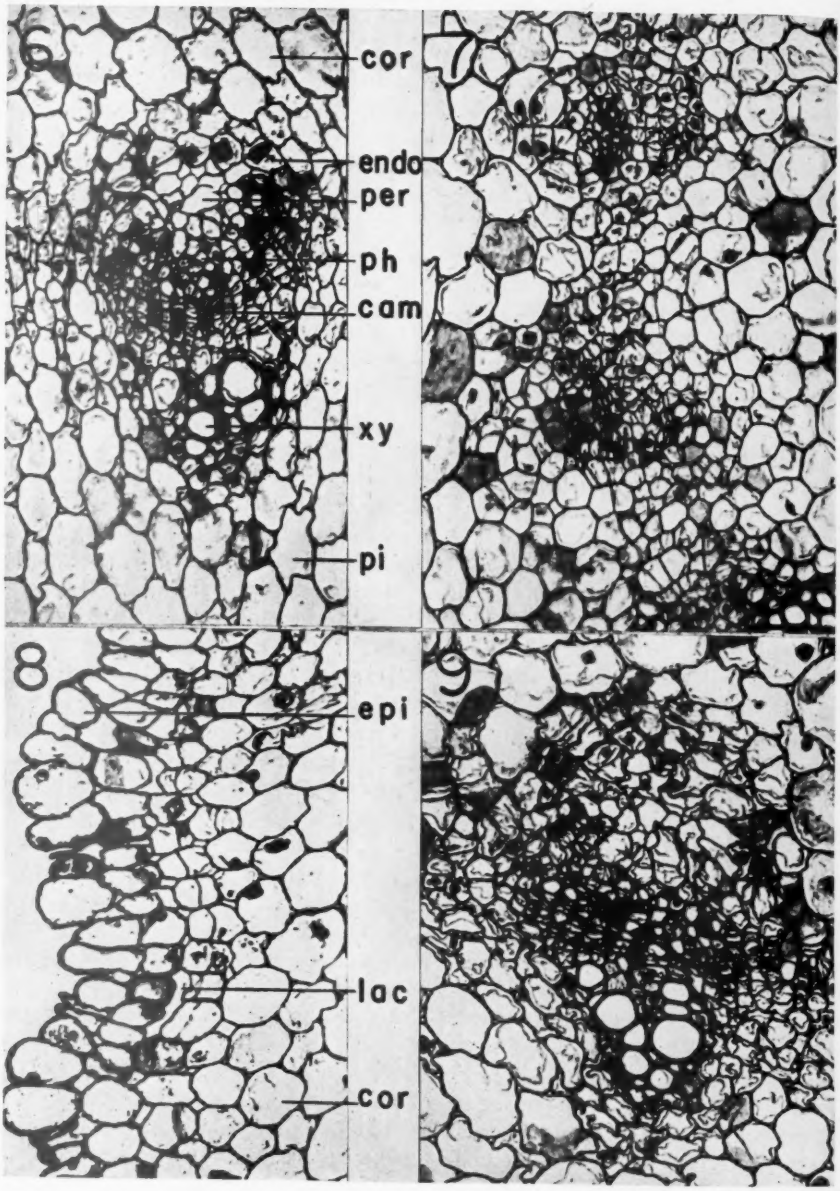
FIGS. 4, 5.—Fig. 4, 0.6 mm. below surface; portion showing leaf trace and cortical strand of fibers 96 hours after treatment with lanolin alone. Fig. 5, 0.9 mm. from surface; portion showing cortical stipular bundle 56 hours after treatment with lanolin alone. *epi*, epidermis; *lac*, lacuna; *cor*, cortex; *pi*, pith; *per*, pericycle; *per f*, pericyclic fibers; *endo*, endodermis; *ph*, phloem; *cam*, cambium; *xy*, xylem; *r*, ray.

the cells of which are not clearly differentiated but which is easily recognizable in fresh sections stained with iodine because of the presence of stored starch. Associated with each larger bundle is a small group of pericyclic fibers, largely immature, often separated from the phloem by a few parenchymatous cells. A fascicular cambium is usually present in the larger bundles but not always in the smaller ones. With increased age a fascicular cambium develops in the smaller bundles, and an interfascicular cambium, which may give rise to a few derivatives, forms between the bundles. The cortex consists of parenchymatous cells, rounded in cross-section, with intercellular spaces. The outer four or five layers of cells contain chloroplasts. In the outer part of the cortex between the first and second layers under the epidermis small lacunae are present. The epidermis is made up of rather small cells, rounded or radially rectangular in cross-section, and is covered with a thin layer of cutin. It contains numerous stomata, and there are a few glandular hairs. The cells of the pith are parenchymatous and rather large. Usually there is complete disintegration of the innermost ones, leaving the stem hollow.

The initial changes following treatment with indoleacetic acid are so gradual that it is difficult to determine when they first begin. By 30 hours after treatment, however, certain characteristic differences can be seen (fig. 3). The epidermal cells and outer layers of the cortex have enlarged in radial dimension. Chloroplasts have almost entirely disappeared from the enlarged cells. The enlargement causes the margin of the stem, which in an untreated plant or in one treated with lanolin alone is regular in outline, to be thrown into convolutions. At the same time the cells of the cambium have become somewhat more active. The region of undifferentiated derivatives is slightly wider, and the nuclei appear larger. In the parenchymatous cells of the cortex, rays, and pith, activity appears to be somewhat increased, as some of the nuclei are larger and there are more division figures observable than in a stem treated with lanolin alone for the same number of hours.

Development subsequent to this beginning is shown in figures 6–23. Although plants were collected at frequent intervals following the appearance of early differences, only those were photographed which showed a definite increase in response. All plants did not respond at exactly the same rate. Differences can be associated with the amount of lanolin mixture spread on the surface, with the maturity of the tissues at the time of treatment, and probably also with growing conditions at that time. Changes were initiated sooner and development proceeded more rapidly in those plants that were highly vegetative at the time of decapitation.

The changes observable at 30 hours increase with length of time after treatment, both in amount of response in regions already referred to and in number of regions responding. At 36 hours some of the endodermal cells have increased slightly in size, but more striking than this is the increase in size of the nuclei and the initiation of their division (fig. 6). An occasional endodermal cell divides in a tangential plane. In the region where this activity begins, just below the cut surface, starch has disappeared but is still present farther away from the surface, where activity in the endodermis has not yet begun. Early divisions of the endodermis always appear in connection with a vascular bundle, either large or



FIGS. 6–9.—Fig. 6, 0.95 mm. from surface; 36 hours after treatment, showing enlarged nuclei of first division of endodermis outside a bundle. Fig. 7, 1.05 mm. below surface; 52 hours after treatment, showing divisions in endodermis, formation of interfascicular cambium, and increase of cytoplasm. Fig. 8, 0.4 mm. from surface; 38 hours after treatment, showing tangential divisions in outer cortex. Fig. 9, 0.8 mm. below surface; 92 hours after treatment, showing repeated divisions in two planes within one endodermal cell forming provascular strand.

small. After 40 hours one division and occasionally a second have occurred in cells around the bundles; after 48 hours almost all the endodermal cells over bundles have begun to divide (fig. 7); after 72 hours the greater part of the derived endodermal layer is three or four cells thick; while at 80 hours divisions are beginning in the endodermal cells between the bundles, forming an almost continuous region of proliferated tissue around the stem. The extensions of the endodermis around the leaf traces, whether including the cortical fiber bundle or not, are evident (fig. 10). Between 84 and 92 hours the derivatives of one, sometimes two, endodermal cells undergo a series of divisions in various planes without subsequent enlargement, forming a compact group of small, very active cells (fig. 9). They appear to be provascular strands. The endodermis and cortex around the cortical fiber bundle divide, at first tangentially and later in several planes, forming provascular strands.

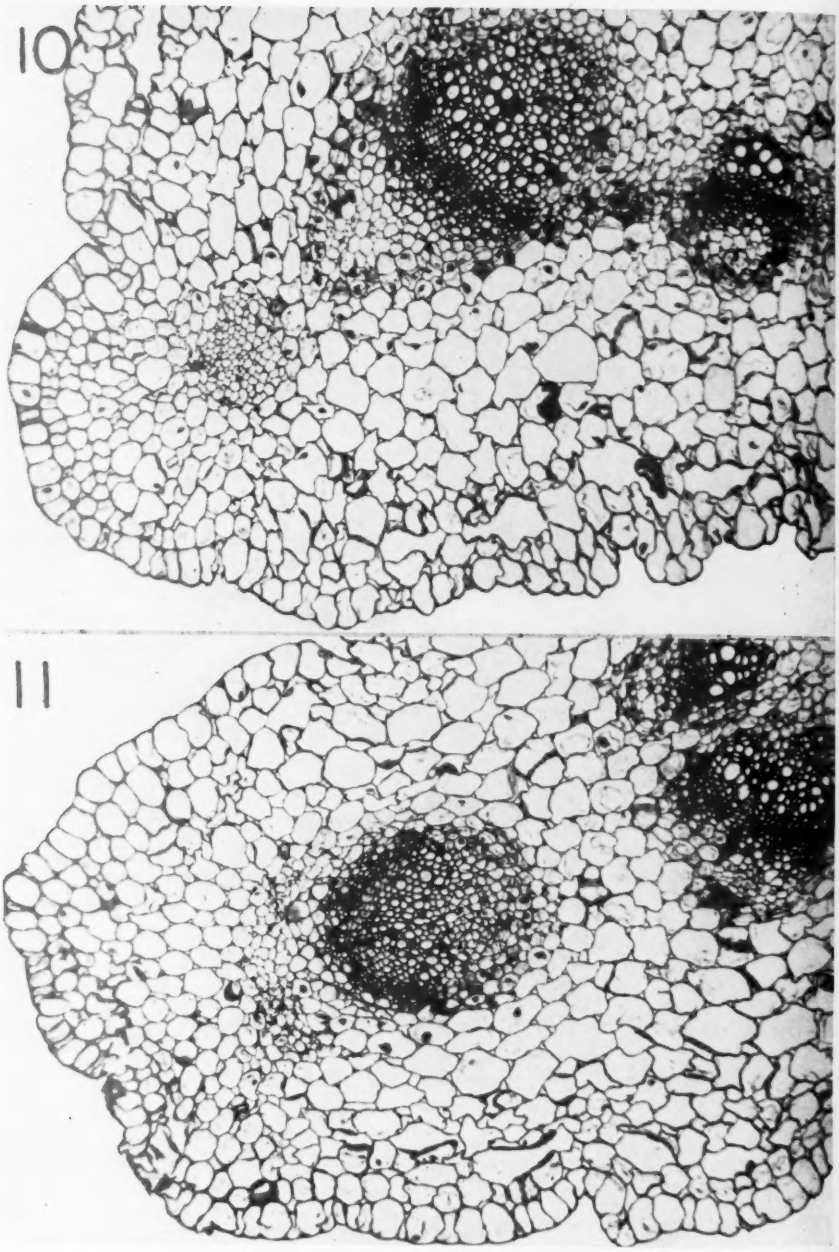
In a longitudinal section of a tumor 72 hours old the endodermis can be followed from a layer one cell wide at the surface to a layer three, four, or five cells wide immediately below the surface, and then narrowing again below, but more gradually, to a single layer. Portions of the endodermal cells sometimes appear to project into the pericyclic fibers next to them, similar to the projections of parenchymatous cells into vessels forming tyloses. Nuclei migrate into the projections and there undergo division.

After 40 hours, in some places in a few stems tangential divisions can be seen in the second cell layer of cortical parenchyma under the epidermis (fig. 8). This type of division does not appear at all consistently but is found occasionally in tumors of varying ages. It may perhaps be associated with the presence of a little lanolin mixture on the side of the stem as well as on the surface. In spite of care to put the mixture only on the decapitated surface, occasionally a little may get on to the side.

The amount of cytoplasm in the active cells tends to increase gradually after 36 hours but never becomes as dense as it does in bean. Activity in the cambium increases with length of time after treatment, older stems showing a noticeable development of secondary xylem. At 40 hours there are indications of the beginning of an interfascicular cambium, which become more evident after 48 hours (fig. 7). At 72 hours it is rather well developed, and at 80 hours its activity has almost completed the vascular cylinder (fig. 10).

By 48 hours after treatment cells scattered through cortex and pith show nuclei in various stages of division, together with cells which appear to have recently divided. These scattered divisions are chiefly transverse as seen in longitudinal section. Transverse divisions would account for the rather large number of mitotic nuclei observed in cross-sections, in contrast to the relatively small number of recently divided cells observed in cross-sections of even older tumors (up to 100 hours). They would also account for the increase in number of nuclei visible in cross-section of a treated stem, as the resulting cells are shorter in longitudinal dimension.

Parenchymatous cells of the phloem may show slight activity after 48 hours. Ray cells sometimes divide, particularly transversely, in the early stages. A longitudinal section may show some cells with two or three nuclei and no cross walls, but this is rather infrequent. Cambium, xylem, phloem, and ray parenchyma are active in early callus proliferation when callus is formed. By 72 hours in those tu-



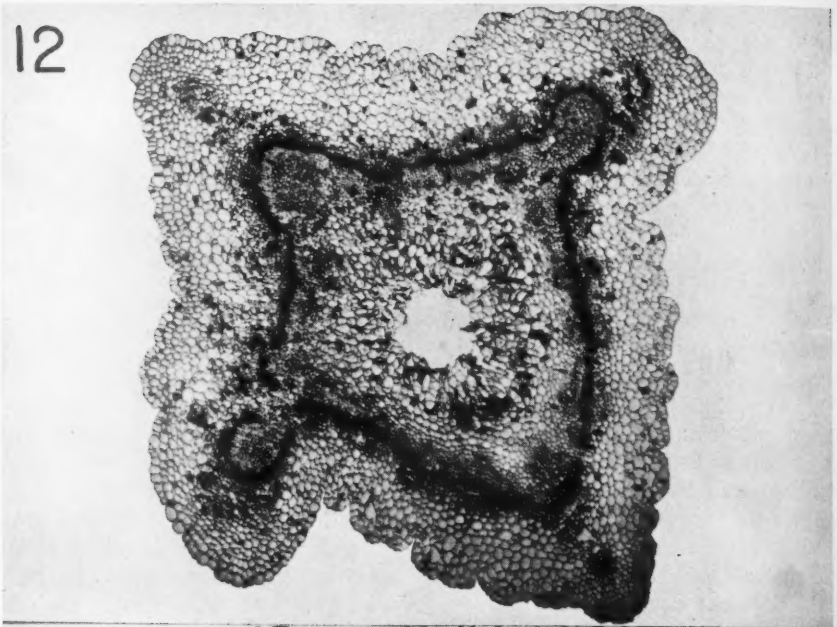
FIGS. 10, 11.—Seventy-two hours after treatment. Fig. 10, 1.1 mm. below surface; activity in endodermis around leaf trace; cortical strand of fibers outside central cylinder. Fig. 11, 1.6 mm. below surface; stipular bundle becoming active at inner edge.

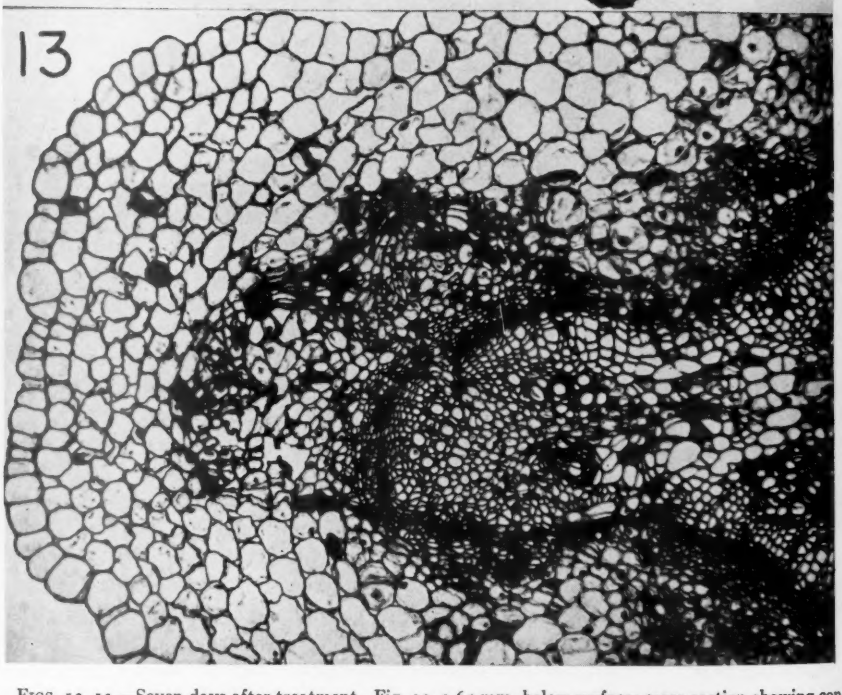
mors which do form callus the living cells just below the cut surface have begun to elongate upward and to divide, beginning the formation of a callus over the surface. In much older tumors other parenchymatous cells also become active and add to the proliferation.

By 72 hours parenchymatous cells at the lateral and inner edges of the stipular bundles have become active and show several derivatives (fig. 11). Later such cells between the stipular bundle and the central cylinder may divide. None of the active regions extends more than 2–3 mm. back from the cut surface. The activity in the fascicular and interfascicular cambium, the enlargement of the cortical and epidermal cells, and occasionally the divisions of the endodermis appear farthest from the surface. The largest part of the tumor up to 100 hours after treatment is due to the radial enlargement of the cortex and epidermis.

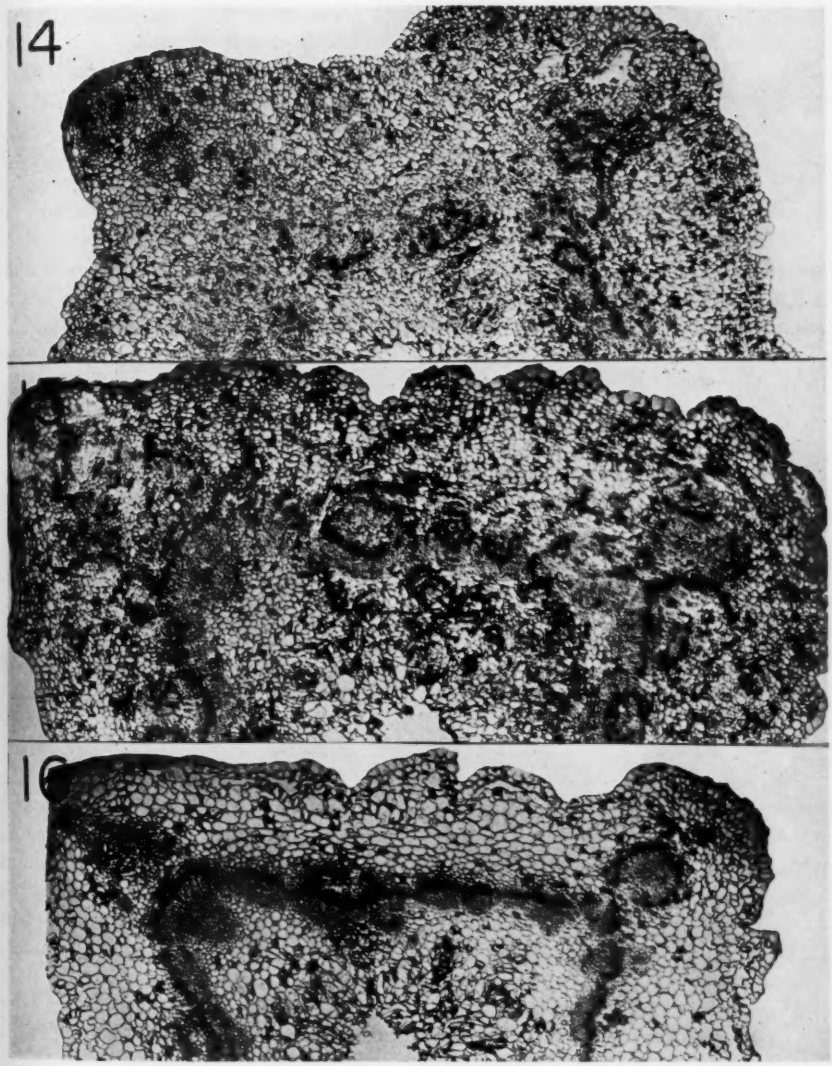
Development of tumors subsequent to the first 100 hours has not yet been followed closely, but later responses will be described briefly from several older tumors of widely separated ages. After 7 days, responses already described have increased. Owing to the continued activity of the fascicular and interfascicular cambium, the vascular cylinder is almost complete around the stem, with a great deal of secondary xylem (fig. 12). Activity of the cells around the stipular bundles, especially laterally, has resulted in a continuation of the cambium, with xylem differentiating on the inner side and phloem on the outer. This development leads to a bundle, horseshoe-shaped in outline, with secondary xylem completely surrounding the primary strand except for a small area nearest the stele (figs. 12, 13). The endodermis has continued activity and in many places shows small vascular strands starting to differentiate. In some places the cortical cells next to the endodermis show an increase in both cytoplasm and divisions (fig. 13). Some of the pericyclic fibers, evidently immature at time of treatment, and some pericyclic parenchymatous cells, have become active and may show several derivatives. The walls of others of the fibers have thickened and remain single, or in small groups, scattered among the active cells. In one or two instances there are a few cells in the cortex, grouped together, which have divided repeatedly to form provascular masses. At this time, however, the largest part of the cortex is unresponsive to the treatment. The pith, which earlier showed little response, has become highly active. The parenchymatous cells bordering the hollow center have proliferated inward, forming a callus in the hollow portion. The cells of the callus and those next to it have divided repeatedly, in many places tangentially and in others in various directions, to form provascular masses.

Tumors a month or more old show a still greater response. A short distance below the cut surface is found the region of greatest activity (fig 15). The surface of application may be covered with a thin layer of callus, in which wound tracheids may have differentiated irregularly (fig. 14). In the active region the proliferated endodermis has given rise to vascular strands. Some cells of the pericycle are active in central cylinder bundles and in stipular bundles. In connection with the leaf traces, cells of the primary phloem have become active, forming vascular strands to increase the amount of extra-vascular tissue already differentiated in this region from pericycle and endodermis. The strands derived from endodermis, pericycle, and phloem are distinct from one another but





FIGS. 12, 13.—Seven days after treatment. Fig. 12, 0.65 mm. below surface; cross-section showing continuous vascular cylinder, large amount of secondary xylem, callus proliferation inward from pith cells, activity in endodermis, beginning activity in cortex (lower left corner). Fig. 13, 0.5 mm. from surface; same stipular bundle as in upper right in fig. 12 enlarged. Bundle does not usually become continuous with central cylinder as shown here. Note vascularization in endodermis, activity in pericycle, activity of inner cortex.



FIGS. 14–16.—Cross-sections at different levels of 35-day-old tumor. Fig. 14, 0.25 mm. from surface; largely callus. Fig. 15, 0.85 mm. from surface; activity of cortex, pith, and vascularization of endodermis. Fig. 16, 2.5 mm. below surface; evidences of activity at this level in pith, cambium, endodermis, and around stipular bundle.

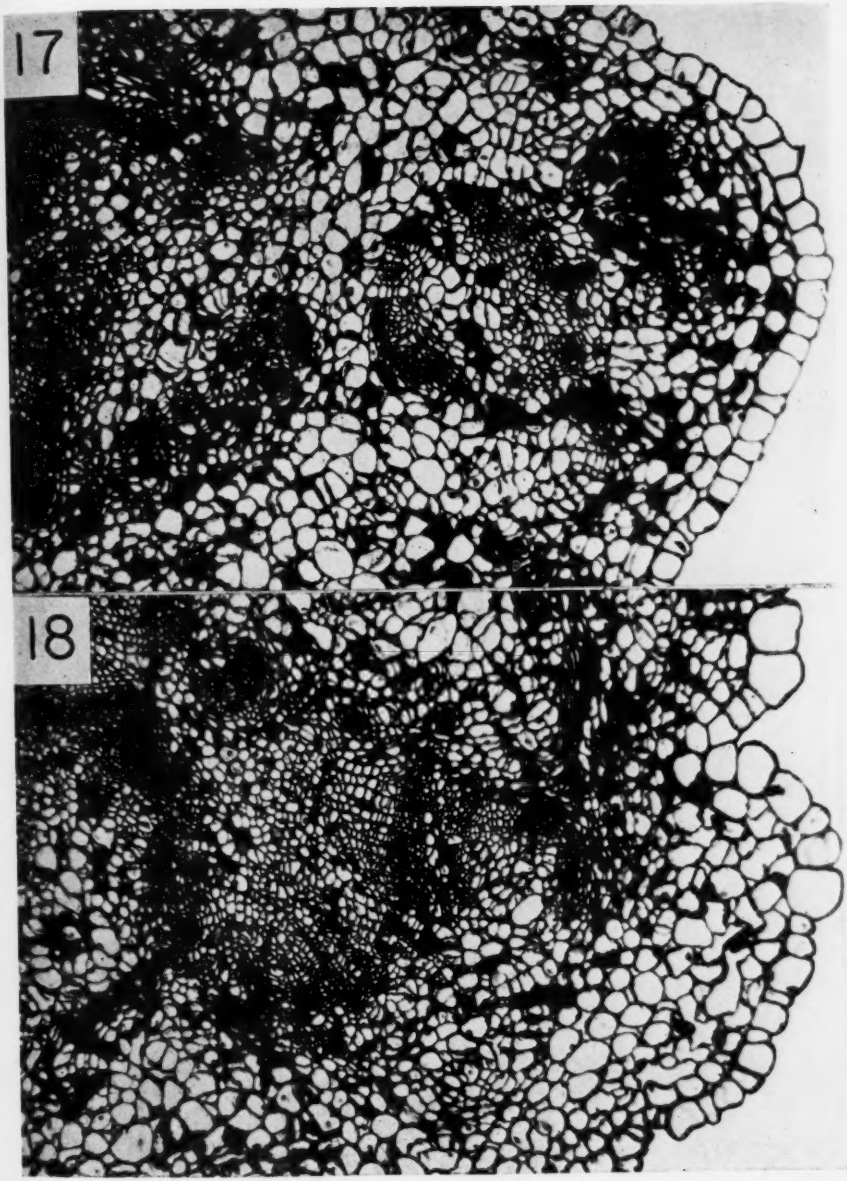
form a zone around the central vascular cylinder (figs. 15, 19, 20), around the stipular bundles joining them to the central cylinder (fig. 18), and around the large zone of pericycle outside the leaf traces (fig. 17). Many of the extra-vascular strands differentiate a cambium which is active in forming xylem toward the center of the stelar region and phloem to the outside. When the endodermis was continuous around the group of pericyclic fibers in the cortex, the extra-vascular strands derived from it are also continuous around the bundle. Strands found between the phloem and pericycle, evidently formed from the phloem, also develop a cambium, but differentiation of its derivatives is the reverse of that in the endodermal strands. Xylem is formed toward the pericyclic mass and phloem toward the phloem of the leaf trace. There is thus formed a complete new vascular ring, largely outside the main vascular cylinder (note leaf trace in the upper lefthand corner of fig. 15). Where the groups of fibers are separate from the leaf trace, the extra-vascular strands are between the cortical group and the leaf trace. The group of fibers, however, is surrounded by endodermis, which has formed—probably in conjunction with cortical parenchyma—vascular strands of the same type as around the leaf trace proper (fig. 17).

Cells of the cortex, both next to the endodermis and just under the epidermis, are active and may show as many as ten or more small cells within one mother cell wall. Vascular masses also differentiate in the cortex. Pith cells become active right up to the vascular bundles and may form vascular strands or masses of tracheids near the xylem of the main vascular cylinder (fig. 19). Callus in the hollow portion of the pith is extensive. Between 0.5 and 1 mm. from the treated surface hardly a single cortical or pith cell remains inactive, and small vascular and meristematic masses are numerous (fig. 15). Occasionally tangential divisions in the epidermis may be found.

Vascular strands in the endodermis (particularly in association with the leaf traces), the horseshoe development of the stipular bundles, the formation of callus from the pith cells, the activity of fascicular and interfascicular cambium with differentiation of secondary xylem, and the enlargement of cortical and epidermal cells—all extend farthest from the cut surface in the old tumors (figs. 16, 20). Division figures are scarce in old tumors. Apparently continued active proliferation has stopped.

In all the tumors cut, only three showed evidences of root formation. In only one were true root primordia located. This tumor had two primordia occurring at distances of 2 and 3 mm. below the cut surface. They show differentiation into epidermis, cortex, and stele and have a protective cap over the tip, but the histogenic region is not clear. They extend a short distance into the cortex from the vascular region (fig. 21). As these are the only organized roots found and no initial stages have been observed, it is extremely difficult to be sure from what tissue they are derived. As far as can be ascertained, they are formed in the region of a ray of the central cylinder. The endodermis may be involved in part in the formation, but probably the greater portion is derived from cells inward from it.

In addition to these organized root primordia, several peculiar structures were observed. In one tumor 7 days old one small and two large masses of proliferated tissue appear a short distance back from the treated surface. The masses consist of small cells with a great



FIGS. 17, 18.—Thirty-five days after treatment. Fig. 17, 0.85 mm. from surface; leaf trace showing separate vascularization of endodermis around cortical group of fibers. Note activity and groups of wound tracheids in cortex. From same section as fig. 15 from portion not shown in fig. 15. Fig. 18, 0.85 mm. from surface; enlargement of upper right corner of fig. 15; stipular bundle showing extra-vascular bundles joining bundle to central cylinder. Note activity of cortex.

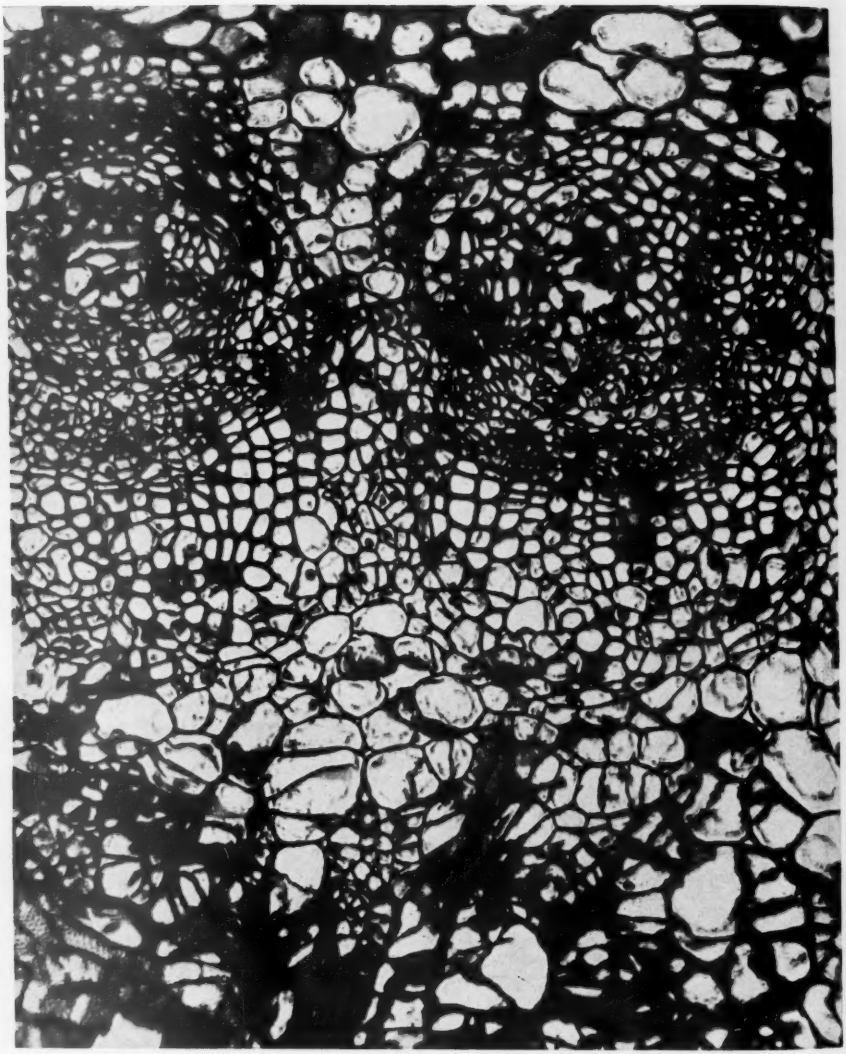


FIG. 19.—Thirty-five days after treatment, 0.85 mm. below surface; enlargement of two small bundles shown in fig. 15. Small bundles of central cylinder showing extra-vascular strands of endodermis, activity of phloem, ray, and pith; thickenings on wound tracheids in pith.

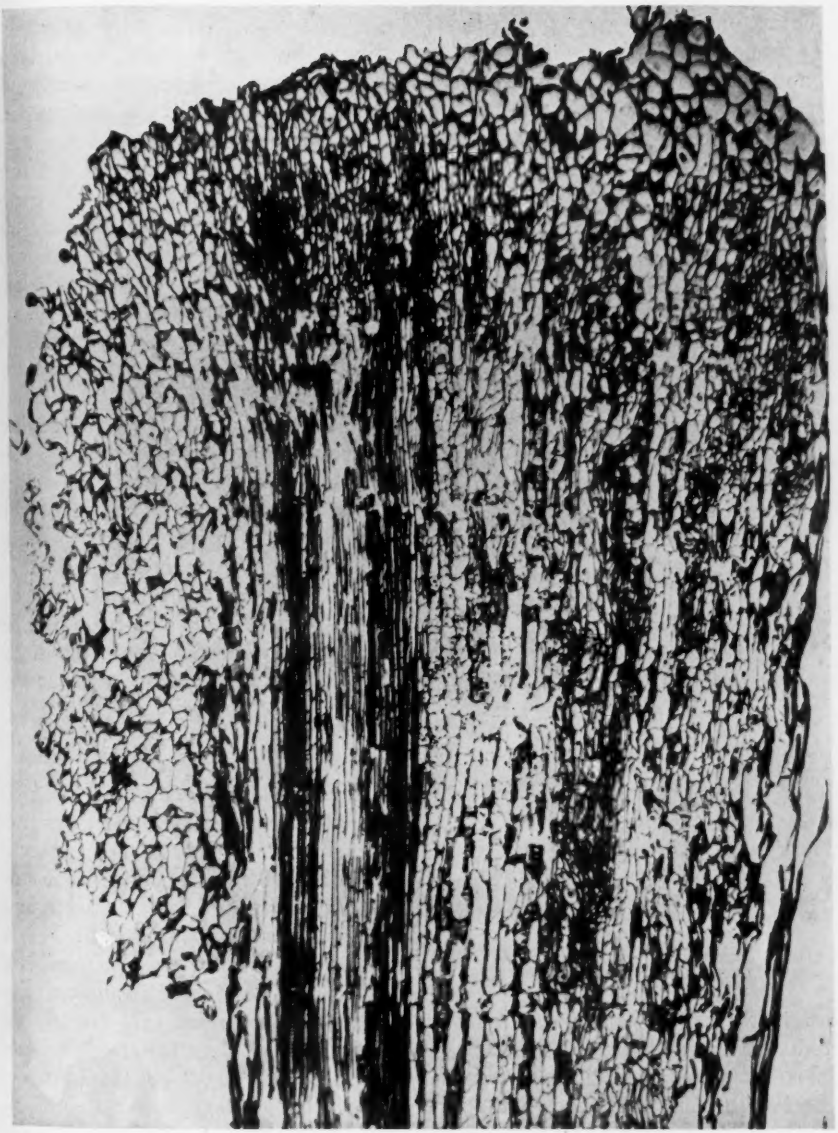


FIG. 20.—Diagonal section of 35-day-old tumor through leaf trace, showing strand in endodermal region, proliferation in cortex, activity of phloem and pericycle, and both pith and surface callus.

deal of cytoplasm and large nuclei. The cells are in active process of division. The group of cells shows no organization of any kind. It has not been possible to determine their derivation accurately, but the endodermis appears to have been

FIG. 21.—Longitudinal section of root primordium 2 mm. from surface of 46-day-old tumor.

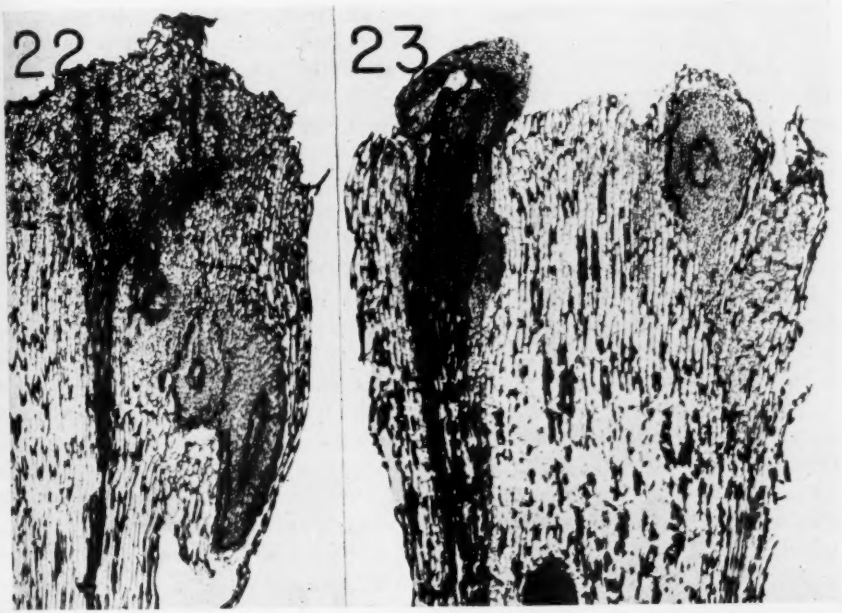
particularly active. In two older tumors proliferations having no particular organization were found. They show differentiated tracheids in the region adjacent to and in contact with the vascular strand of the stem; the differentiated tracheids are continued by regions of smaller cells almost to the edge of the proliferation. The cells are not meristematic; their cytoplasm is highly vacuolate. They may be derived from cells in close association with the leaf trace or stipular bundles, or from cells in the region of a ray of the central cylinder.

The shapes and sizes of the proliferations are various, but tend toward a rounded, somewhat elongated structure, with a suggestion of a ring of differentiated cells, which in many cases are vascular—at least near the bundle with which union is established. They extend upward above the callus surface (fig. 23), laterally through the cortex, or laterally and downward in the cortex (fig. 22). The interpretation suggested is that they may have originated as unorganized meristematic masses of tissue, as in the 7-day-old tumor. Differentiation took place in the direction of a root primordium but did not progress far. With later growth of the tumor the cells did not remain meristematic, but most of them enlarged, became vacuolate, and a few became lignified, finally resulting in the rather unorganized masses of tissue seen in the two old tumors.

CYTOLOGICAL DETAILS

Almost without exception, nuclei of the endodermis, pericycle, cambium, and parenchymatous cells of the vascular bundle and of the cortex and pith in close association with the vascular tissue, and, with occasional exceptions, other cells of the cortex and pith, are of normal size. If in division, they show the diploid number of chromosomes, 12. The normal resting nucleus has two medium-sized nucleoli which may fuse to form one large one. The stages of mitosis all proceed in a normal manner. In a few instances, as already stated, nuclear division apparently is not accompanied by cell plate formation, thus giving rise to binucleate and, in a very few cases, multinucleate cells. In some of the large par-

enchymatous cells of the pith and cortex of both treated and control stems (although somewhat more frequently in the treated) large nuclei are found which may have one very large nucleolus or two large or four medium-sized ones. These number of polyploid cells, the fact that they are found in controls as well as in treated stems, and the regions in which they occur, do not as yet indicate any particular significance to the polyploid condition observed in this experiment.



FIGS. 22, 23.—Proliferations in old tumors. Fig. 22, 35 days old, diagonal section; portions of three proliferations at different distances from stipular bundle. Fig. 23, 46 days old, tangential section; region of cortex, stipular bundle on left. Mass at right establishes union with leaf trace. Portion of cross-section of root primordium at base.

facts, according to BERGER (4), suggest a tetraploid condition. Division figures found in comparable cells show more than the diploid number of chromosomes. In a very few instances the degree of polyploidy may be greater, but this is highly infrequent. These cells with tetraploid nuclei are few, are found in regions not extremely active, and appear to occur normally in the stems of control plants to a certain extent. So few were found that it cannot be said by what method doubling occurred. The small

Discussion

The responses just discussed enlarge upon and corroborate to a certain extent the work of LAIBACH (19) and SOLACOLU *et al.* (27) on *Vicia faba*. LAIBACH reports roots only in the largest callus proliferations formed on decapitated stems treated with indoleacetic acid in lanolin, while according to SOLACOLU *et al.* similarly induced tumors 9–10 days old show root meristems, completely individualized, producing rootlets with limited

growth and abnormal development. The latter find that the tumor extends much farther down the stem than here reported, and that meristematic activity, except in direct contact with the paste, is localized in the meristematic zones, the cambium and pericycle. The results of the present experiment do not agree completely with this. Activity in the cortex extends at least half the length of the older tumor, activity of the pith extends sometimes the complete length of the tumor, and the endodermis is more meristematic in all regions than is the pericycle. They report extreme activity near the surface in older tumors but give no account of the vascularization of the endodermal derivatives reported in the present paper. They do, however, describe many tracheids differentiated in various regions near the cut surface.

The responses of *V. faba* to indoleacetic acid show marked similarities to the responses of other plants to similar treatments, although there are certain differences, notably in degree of response. As stated by HAMNER and KRAUS (13) for kidney bean, the results of this experiment indicate that any parenchymatous tissue of the stem of broad bean may become meristematic in response to treatment with indoleacetic acid and these cells later differentiate into vascular tissues. The epidermis forms an exception to this, but in old, very active tumors of *Vicia*, some epidermal cells may undergo division.

The slight surface callus formation of *V. faba* when treated with indoleacetic acid is different from the reaction of kidney bean (13), cabbage (9), tobacco (11), and sunflower (5) to the same treatment, while resembling somewhat the response of tomato (6), *Iresine* (14), pea (26), and *Mirabilis* (12). The regions of greatest activity vary from plant to plant and also with the growth substance used. The extreme activity of the cortex of broad bean resembles that of the kidney bean treated with naphthaleneacetic acid, except for the vascularization (13). Meristematic activity of the endodermis is found in many of the plants that have been treated; it is more extended in kidney bean (2, 13, 17, 22) than in broad bean. Increased activity of the fascicular cambium and development of an interfascicular cambium as seen in broad bean are characteristic of tumors of *Mirabilis* (12), and to a certain extent other plants treated with indoleacetic acid; but greatest development is reached, particularly as regards production of secondary xylem, in tumors of kidney bean induced by naphthalene acetamide (18). The pericycle, which is rather inactive in broad bean, tomato (6), cabbage (9), kidney bean (13, 17), and sunflower (5), is a region of great activity in *Mirabilis* (12) and *Iresine* (14), and is relatively active in pea (26). The phloem, which in broad bean is relatively inactive, is very active in kidney bean (2, 13, 17), pea (26), *Iresine* (14), and tomato (6). Divisions in the pith, so marked in *Vicia* tumors, are also found in others, as in *Mirabilis* (12), to a limited extent in tomato (6), in the formation of the callus in kidney bean (13), in limited regions of cabbage (9), and in sunflower (5). In other plants the pith remains relatively inactive, except close to the primary xylem strands. The relative inactivity of the epidermis is characteristic of all plants treated with indoleacetic acid, except of laterally treated stems of *Iresine* (14).

Irregular differentiation of vascular tissues is found in many of the tumors, but vascularization to the extent and

regularity characteristic of *V. faba* is not found except in the kidney bean tumors induced by tryptophane (16). The similarity between these two tumors in this and other respects is particularly interesting and needs further detailed consideration.

The greatest difference between the tumors produced by *V. faba* after treatment with indoleacetic acid and similarly produced tumors on other plants is the almost complete lack of root initiation. Kidney bean tumors induced by tetrahydrofurfuryl butyrate (25) and tryptophane (16) also show no root formation, but in other indoleacetic acid-induced tumors root formation is extensive and a characteristic feature of the tumor. The rather unorganized proliferations of *Vicia* correspond to nothing reported for other plants, unless such proliferations are interpreted as roots. If this is done the problem remains as to why the cells should have become vacuolate and nonmeristematic without complete differentiation and organization of tissues characteristic of roots. In other plants the root primordia, even though they may not develop far enough to break through the epidermis, remain meristematic and will resume activity if placed in a moist atmosphere. The occurrence of these structures leads to speculation as to the possibility of the existence of an organizing substance not present—or not produced in sufficient quantity—in *V. faba* for the development of normal roots.

The results of the present work, when compared with other similar work, would seem to suggest that both the degree and the type of response to any one growth-regulating substance, such as indoleacetic acid, varies with the species, each species offering a somewhat different internal environment. Certain of the investigations indicate that the effects vary in degree, particularly with variation in external environment, and in degree also with the age of the plant at time of treatment. This latter case has also proved true in *Vicia*.

Although polyploidy occurs to some extent in the tumors produced on the stem of *V. faba*, it seems improbable that it is specifically induced by the indoleacetic acid, although GREENLEAF (11) considers the polyploid condition of shoots produced by decapitated tobacco plants to be induced by this acid and LEVAN (20) finds polyploid cells in abundance in roots of onion treated with indoleacetic acid and other growth substances. DERMEN (8) reports polyploid cells in bean stems treated with naphthaleneacetic acid, but these are mainly in tissues likely to disintegrate subsequently. The potentiality for polyploidy is present normally in stems of *V. faba*, as shown by the scattered large nuclei and occasional polyploid figures in sections of control plants. It is conceivable that this tendency receives greater expression in treated plants because of the delay in maturation of and stimulation to increased activity in the cells in which the polyploidy occurs. A similar type of explanation might be extended to the case of tobacco, where callus does not normally form (10) but is induced by indoleacetic acid[1] and to the case of bean treated with naphthaleneacetic acid. The appearance of great numbers of polyploid cells in onion roots is more difficult to explain on a similar basis, although great numbers of such cells are found normally in roots of certain plants,

[1] Tomato produces polyploid shoots from a callus without the presence of the growth substance (21, 23).

for example, *Spinacia* (3), and moderate numbers in roots of certain legumes (28). However that may be, much more careful study is necessary before the specific relationship between growth-regulating substances and the possible induction of polyploidy can be established.

Summary

1. Plants of *Vicia faba* were decapitated just below the fifth node and the cut end treated with 1.5 per cent indoleacetic acid in lanolin or with lanolin alone.

2. The plants treated with indoleacetic acid responded by the formation of a tumor. The area, at most not more than 5 mm. long, below the treated surface became swollen and yellowed. There was little increase in size after the first 2 weeks following treatment.

3. The anatomy of the fourth internode of *V. faba* is similar to that in *Pisum sativum*, except for the number of bundles in the central cylinder.

4. Histologically every parenchymatous tissue became active to some extent in the formation of the tumor. Epidermal and outer cortical cells enlarged radially; the inner cortical cells divided transversely, occasionally the outer cortical cells tangentially; the endodermis divided tangentially; the cambium increased its activity and an interfascicular cambium was formed and became active as the early signs of response. Later parenchyma of the xylem, phloem, pericycle, pith, and rays became active to varying degrees. Cells of the pith divided repeatedly, and those bordering on the central cavity gave rise to an internal callus, the cells of which also divided repeatedly. In later stages all the cells of the cortex became highly meristematic. In some cases a callus was formed above the cut surface, starting from active cells of the vascular region and added to eventually by all parenchymatous cells. In the oldest tumors an occasional tangential division of the epidermis may be found.

5. Vascularization of derived tissues is somewhat extensive. Particularly notable is the response of the endodermal region of the central cylinder and of the cortical bundles. This group of extravascular strands may be supplemented by an occasional one formed in the pericycle or phloem. Small groups of wound tracheids are differentiated in the pith, pith callus, cortex, and surface callus when present. Occasionally vascular strands are formed in the pith close to the protoxylem points of the regular vascular cylinder.

6. Root induction, characteristic of some other plants treated with indoleacetic acid, is uncommon in *V. faba* under the conditions of this experiment. Those formed are probably derived as described for other plants. Occasionally, however, large, rather unorganized masses of tissue are developed which are probably best interpreted as potential roots incompletely organized.

7. Occasional polyploid nuclei are found in the large parenchymatous cells of the cortex and pith in both treated and control stems of *V. faba*, although somewhat more numerously in the treated stems. All divisions appear to be regular.

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STUDIES IN CALIFORNIAN HEPATICAE
III. CRYPTOMITRIUM TENERUM

ARTHUR W. HAUPT

(WITH TWENTY-EIGHT FIGURES)

Introduction

Cryptomitrium tenerum (Hook.) Aust. is said by EVANS (4) to occur in "California and Mexico; probably also in Chili." Since this was written, the species has been reported from Washington by CLARK and FRYE (2), while more recently it has been collected by the writer (9) in Costa Rica and Guatemala. Its occurrence in South America is doubtful, being based on its supposed identity with *Duvalia brevipedunculata* Mont. from Chili, described nearly a century ago. *Cryptomitrium* was long thought to be monotypic, but a second species, *C. himalayense*, has been described from northern India by KASHYAP (11).

Cryptomitrium tenerum is widely distributed in California, but, at least in the southern part of the state, seems nowhere to be locally abundant. It has been collected by the writer in the Santa Monica, San Gabriel, and Cuyamaca Mountains at relatively low elevations, mostly below 2000 feet, where it grows in ravines on moist shaded banks. It usually reaches its best development in February or March, depending on the amount and distribution of the winter rainfall. During the long rainless season the plants dry up, the tips reviving and giving rise to new plants with the advent of the autumn rains.

Material for the present study was collected both in southern California and in Costa Rica. No differences were observed between the plants obtained from these two widely separated regions. They undoubtedly belong to the same species. The superficial features of *C. tenerum* are well described and illustrated by HOWE (10). The only morphological investigation that has been made is by ABRAMS (1). *Cryptomitrium* belongs to that section of the Marchantiaceae designated by LEITGEB (12) as the Operculatae and which includes the well-known genera *Plagiochasma*, *Grimaldia*, *Reboulia*, and *Asterella*. *Cryptomitrium* is most closely related to *Grimaldia*, differing from it chiefly in having an almost flat rather than a strongly convex carpocephalum and a bilabiate rather than an undivided involucre.

Observations

THALLUS

The thallus is pale green and rather thin and delicate. Its branching is mainly dichotomous but sometimes occurs by means of either apical innovations or ventral adventitious shoots. The epidermal cells, forming a single layer, have slightly thickened walls and many chloroplasts. The air pores are small, simple, and with unthickened radial walls. The air chambers, occurring in several layers, are separated by partitions one cell thick. They are sparingly subdivided by supplementary partitions. Special chlorophyllose filaments are wanting. Chloroplasts are abundant in the cells of the loose dorsal region and also occur in those lying immediately below. The ventral

region is composed of compactly arranged, thin-walled cells. No mucilage cells and no endophytic fungus were seen in the material studied. Oil cells are of infrequent occurrence. The ventral scales, arranged in two longitudinal rows, have filiform appendages. Both smooth-walled and tuberculate rhizoids are present.

Growth of the thallus results from the activity of a single apical cell of the

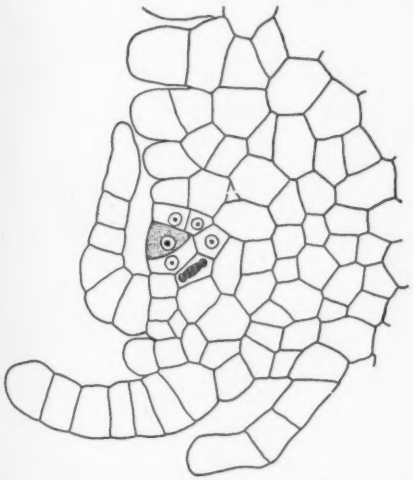


FIG. 1.—Median longitudinal section through apical cell showing schizogenous origin of air chambers behind it. ×450.

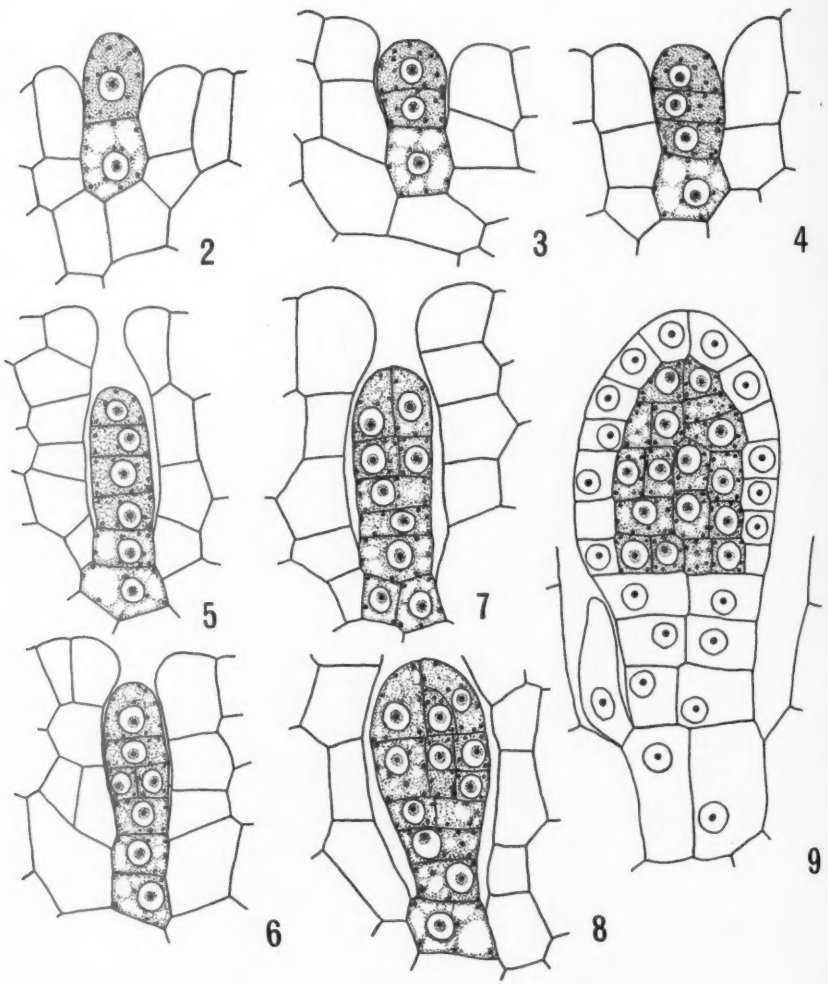
cuneate type, characteristic of all the Marchantiales (fig. 1). The air chambers originate close behind the apical cell by intercellular cleavage, the split beginning at the surface and progressing inward, as in *Reboulia* (5) and *Asterella* (8). The air chambers are deepened by upgrowth of the surrounding tissue.

Cryptomitrium tenerum is monoecious. As in certain species of *Grimaldia* and *Asterella*, the antheridia form a narrow, elongated, median, dorsal group lying immediately posterior to the female receptacle. The pore communicating with the antheridial chamber is considerably raised above the general level of the thallus. The air pores communicating with the surrounding air chambers are simple, as in the other genera of Operculatae.

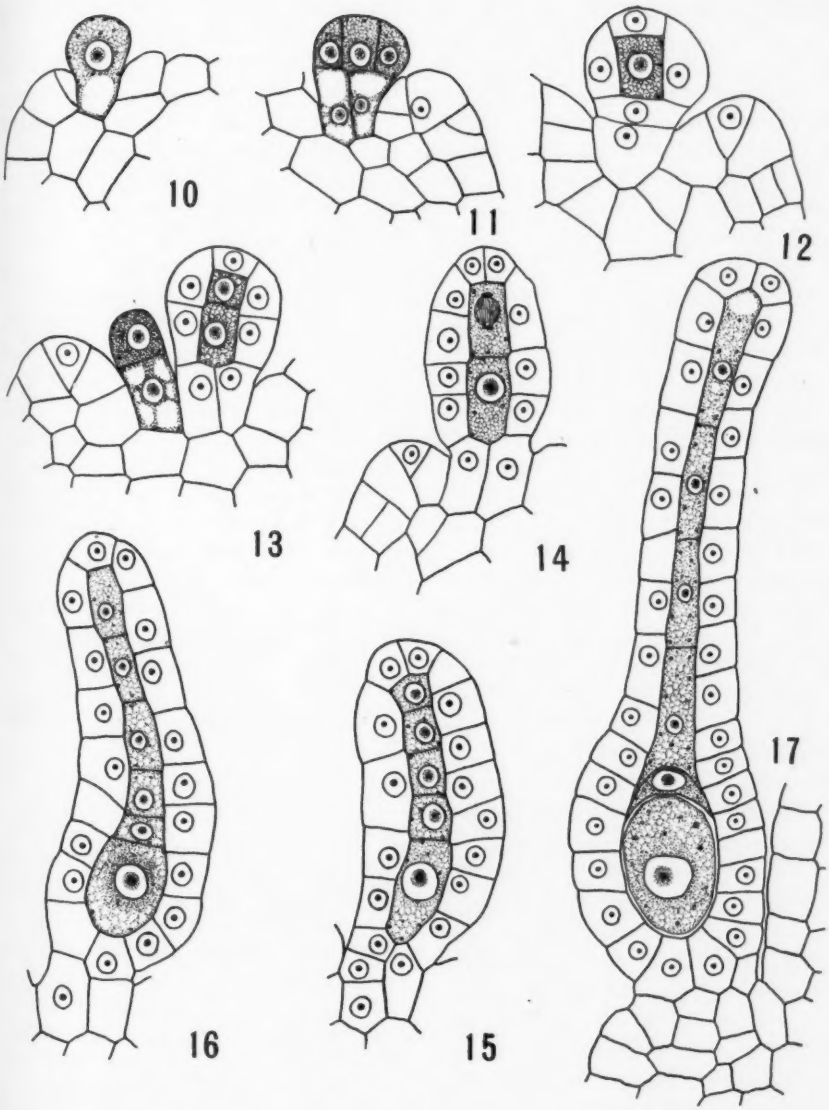
The female receptacle is terminal. Its stalk has a single rhizoid furrow. The disk is hemispherical when young but finally becomes circular and nearly flat. It is without lobes when mature. ABRAMS (1) has demonstrated that the young receptacle has five growing points (rarely four) and thus represents a branch system. The apical cell of the thallus is carried upward on the receptacle and soon undergoes two successive dichotomies. Of the four growing points thus established, one usually divides again to form five in all. Between the growing points lies a rhizoid furrow continuous with the furrow of the receptacle stalk. Three or four archegonia arise in acropetal succession immediately behind each growing point. They become surrounded by a bilabiate involucre. The activity of the apical cells on the carpocephalum is checked by the formation of the archegonia, further growth being intercalary. As in the other genera of Operculatae, the air pores on the female receptacle are compound (barrel-shaped) but elsewhere are simple. The air chambers on the female receptacle are unusually extensive. They form a single layer.

ANTHERIDIUM.—The antheridia arise in acropetal succession from immediate dorsal segments of the apical cell. They become sunken in individual pits in the thallus formed by upgrowth of the surrounding tissue. The papillate initial undergoes a transverse division into an inner and an outer cell (fig. 2). Generally three additional transverse divisions in the outer cell result in the formation of a filament of four cells in the superficial portion of the antheridium before any

vertical divisions take place (figs. 3–5). Vertical walls appearing at right angles to each other are developed in the three uppermost cells, while another transverse wall may be formed in one of the cells below (figs. 6, 7). Periclinal walls arising in the three uppermost tiers now cut off the outer sterile jacket from the central spermatogenous cells (figs. 8, 9). The lower cells form the stalk, which in the mature antheridium becomes rather thick. As in *Reboulia*, unicellular hairs arise at the



FIGS. 2–9.—Stages in development of antheridium: fig. 2, first division of antheridium initial; figs. 3–5, division of outer cell to form filament of four cells; figs. 6, 7, formation of vertical walls; figs. 8, 9, formation of periclinal walls. ×900.



FIGS. 10–17.—Stages in development of archegonium: fig. 10, archegonium initial; fig. 11, formation of primary axial cell and primary wall cells; fig. 12, formation of central cell and cover cell; fig. 13, first division of archegonium initial (left) and formation of primary ventral cell and primary neck canal cell (right); fig. 14, division of primary neck canal cell; fig. 15, formation of four neck canal cells; fig. 16, differentiation of ventral canal cell and egg from primary ventral cell; fig. 17, nearly mature archegonium. ×600.

base of the young antheridium and secrete mucilage into the antheridial chamber (fig. 9).

The development of the antheridium follows the usual pattern that has been described in *Reboulia* (5), *Asterella* (8), *Preissia* (7), and other Marchantiales. ABRAMS (1) found in *Cryptomitrium* that the outer cell resulting from the transverse division of the antheridium initial gives rise to a row of three rather than of four cells before any vertical walls are developed. This condition was observed in two instances during the present study but is certainly not the usual one. ABRAMS found also that the uppermost cell of the young antheridium does not contribute to the formation of the spermatogenous tissue, a feature with which the present investigation is in disagreement, as figure 8 clearly indicates.

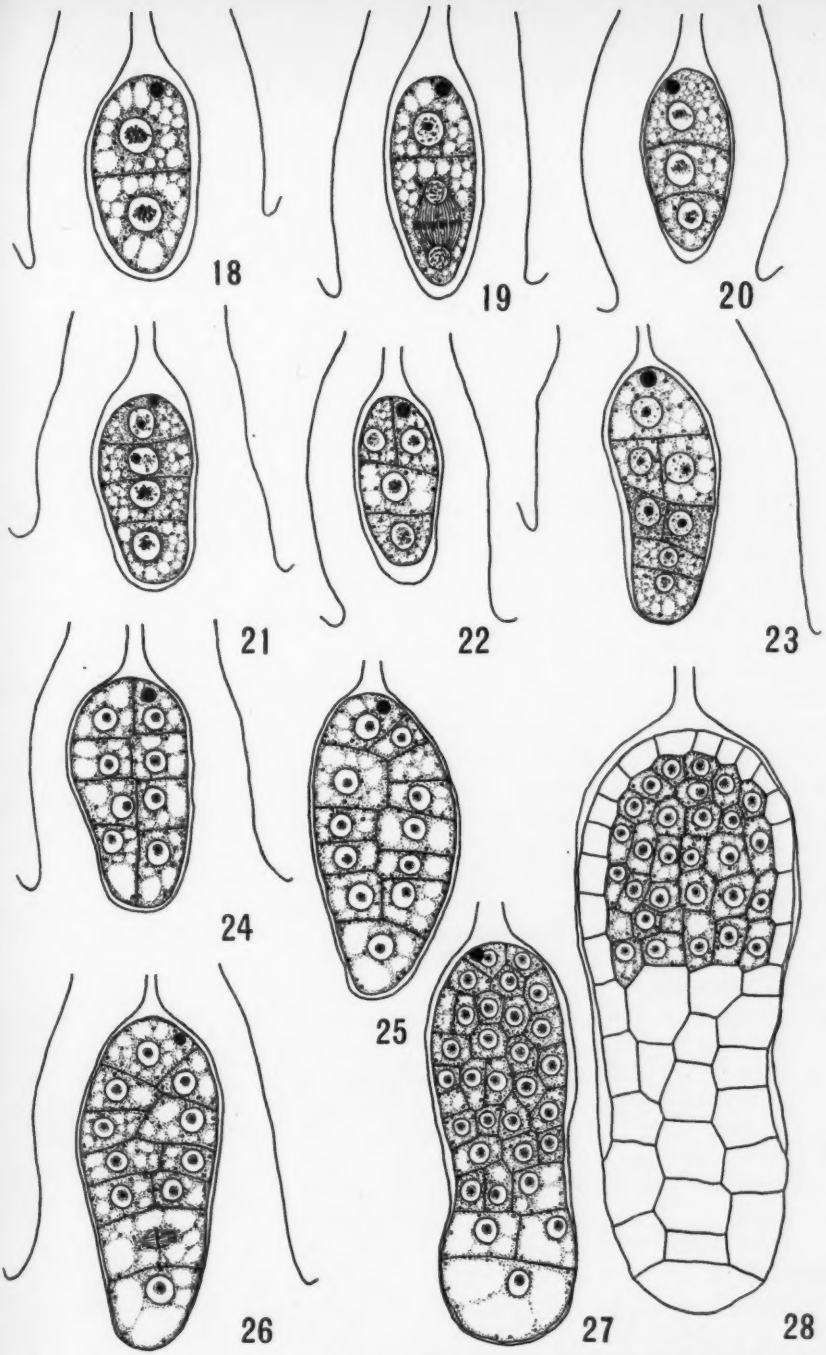
ARCHEGONIUM.—The archegonia appear on the carpocephalum only after it has become conspicuously dome-shaped, arising very close to the apical cell. ABRAMS traced the development of the archegonium and found that the outer cell arising from a transverse division of the papillate initial undergoes the usual three vertical divisions that differentiate the primary axial cell from the primary wall cells. These stages have been confirmed by the present investigation (figs. 10, 11). The primary axial cell gives rise to the cover cell and central cell (fig. 12), the central cell to the primary neck canal cell and primary ventral cell (fig. 13), and the primary ventral cell to the ventral canal cell and egg (fig. 16). The ventral canal cell is small. A vertical wall is formed in the cover cell before the primary neck canal cell divides (fig. 14). ABRAMS observed that this vertical wall does not appear until some time after four neck canal cells have been formed. He also found that "the usual number of neck canal cells is eight."

In all the preparations used in the present study, four neck canal cells are derived from the primary neck canal cell before the primary ventral cell divides to form the ventral canal cell and egg (figs. 14–16). Ordinarily, with the increase in length of the neck and rounding up of the egg, the number of neck canal cells remains four (fig. 17). In many archegonia approaching maturity, however, some or all of the neck canal cells become binucleate. In *Reboulia* (5) the ventral canal cell and egg are differentiated after four neck canal cells have been formed, but this number is later increased to eighteen or twenty. In *Asterella* (8) the situation is similar, except that only eight neck canal cells are eventually produced.

The first archegonia reach maturity while the carpocephalum is still small, its development continuing as additional archegonia are formed. The neck of the mature archegonium hangs straight downward and does not, like that of *Reboulia*, curve outward and upward. It is often slightly twisted. The stalk of the carpocephalum begins to elongate as soon as fertilization has taken place, carrying the embryos upward. Although more than one young sporophyte may be seen in each archegonial group, generally only one (rarely two) reaches maturity. Some of the jacket cells of the venter divide periclinally before fertilization, while after fertilization the venter forms a calyptra that becomes three to five layers of cells thick.

EMBRYO

The first division of the fertilized egg is transverse, forming two segments approximately equal in size (fig. 18). ABRAMS (1) claims that the embryo of



FIGS. 18–28.—Stages in development of embryo: fig. 18, fertilized egg divided transversely; figs. 19, 20, transverse division of hypobasal segment; fig. 21, same of epibasal segment; fig. 22, vertical division of epibasal segment (unusual condition); fig. 23, same of two middle cells and transverse division of basal cell; fig. 24, vertical division of all four cells; figs. 25, 26, older stages with triangular cell at apex and undivided cell at base; fig. 27, older stage showing growth of foot, seta, and capsule; fig. 28, still older stage showing differentiation of sporogenous tissue. ×470.

Cryptomitrium displays an octant stage, but the writer has seen only filamentous embryos. The second division is always parallel with the first one and occurs in the hypobasal segment (figs. 19, 20). Subsequent behavior strongly indicates that, of the three cells formed, the upper one eventually gives rise to the capsule, the middle one to the seta, and the lower one to the foot. Sometimes a vertical division may now occur in the upper cell (fig. 22), but more commonly a transverse division takes place instead, thus forming a filament of four cells (fig. 21). Vertical walls at right angles to each other are then developed in each tier, the four tiers thus consisting of four cells each (fig. 24). This stage was seen frequently. Occasionally an additional transverse division occurs in the basal cell before the formation of vertical walls is complete (fig. 23).

Following the appearance of vertical walls in all four tiers, additional transverse and vertical walls come in somewhat irregularly, especially in the upper two-thirds of the embryo (fig. 27). Few divisions take place in the foot at this stage. Often the walls in the uppermost tier are more or less oblique, so that the embryo seems to have an apical cell, and this may appear to cut off one or more segments (figs. 25, 26). The formation of periclinal walls in the capsular region results in the delimitation of an outer layer of sterile cells from a central mass of sporogenous tissue (fig. 28). Until this stage is reached, a large oil globule is nearly always found at the upper end of the embryo, as in *Reboulia*. In the mature egg several smaller oil globules are generally present.

The early embryogeny of *Cryptomitrium* is similar to that of *Reboulia* as described by WOODBURN (**18**), HAUPT (**6**), and DUPLER (**3**), whose accounts differ chiefly in the interpretation of certain stages found by all. A filamentous embryo consisting of four superimposed cells is formed before the appearance of vertical walls. Both WOODBURN and HAUPT thought that the second wall is developed in the epibasal segment, but DUPLER showed by mitotic figures that the second division occurs in the hypobasal segment and the third in the epibasal segment, a sequence which the present study has demonstrated for *Cryptomitrium*. Furthermore, it seems probable that in *Reboulia*, as DUPLER has suggested, the epibasal cell gives rise to the capsule and the hypobasal cell to the foot and seta. Both WOODBURN and DUPLER observed that the upper cell and occasionally the basal cell of the vertical row of four may divide obliquely to form a triangular cell. DUPLER claimed that the upper one may function as an apical cell.

The writer (**8**) has shown that in *Asterella*, as in *Reboulia*, a filament of four cells is formed before any vertical walls appear. Here also it seems likely that a transverse division occurs in both the hypobasal and epibasal segment, the lowest cell probably giving rise to the foot, the next one to the seta, and the two upper ones to the capsule. In both *Plagiochasma* and *Grimaldia*, MEYER (**13, 15**) has shown that filamentous embryos are produced. In *Grimaldia* the earliest stages were not seen, but in *Plagiochasma* both the hypobasal and epibasal segments undergo transverse division, forming a filament of four cells in which vertical walls are subsequently developed in the usual way. In both genera MEYER observed slightly older embryos with a triangular "apical cell" that seems to cut off a few segments.

In addition to the five genera of Operculatae, filamentous embryos have been observed in *Targionia* by O'KEEFFE (**17**) and in *Conocephalum* (*Fegetella*) by MEYER (**14**). In a discussion of the phylogeny

of the Marchantiales, MEYER (16) regards the filamentous type of embryo as more primitive than the octant type, such as occurs in *Riccia*, *Oxymitra*, *Corsinia*, *Clevea*, *Preissia*, and *Marchantia*.

OLDER SPOROPHYTE

The further growth of the capsule and foot greatly exceeds that of the seta, and as a result the young sporophyte assumes a dumb-bell shape. This is related to the fact that the embryo, filamentous at first, soon becomes considerably elongated. The same condition is seen in the other genera of Operculatae, as well as in *Targionia* and *Conocephalum*. A dumb-bell form is not assumed by the young sporophyte of *Preissia* and *Marchantia*, forms in which the embryo exhibits an octant stage and becomes globular (or but slightly elongated) as further growth and differentiation take place.

The development of the sporogenous tissue in *Cryptomitrium* and its differentiation into spore mother cells and elaters follow the same pattern as that described for *Reboulia* (6). In both genera the spore mother cells and elaters are derived from the sporogenous cells by the same number of cell divisions, and so each elater is homologous with a single spore mother cell. The mature sporophyte has a bulbous foot, a very short seta, and a globular capsule. The capsule wall is one-layered and without annular thickenings, except at the apex, where it consists of two layers, the inner one having much larger cells than the outer one and bearing a few thickenings. The apex of the capsule forms a distinct operculum that remains intact in dehiscence. The elaters have two spiral bands of thickening.

Summary

1. The thallus of *Cryptomitrium tenerum* grows by means of a cuneate apical cell, behind which the air chambers arise by intercellular splitting that begins at the surface.

2. The antheridia are borne in an irregular median dorsal group, the archegonia in a terminal receptacle representing a branch system with usually five growing points, behind each of which three or four archegonia arise in acropetal succession.

3. The air pores on the female receptacle are compound but elsewhere are simple.

4. The antheridia develop in a manner that is typical for the Marchantiales. The formation of periclinal walls delimiting the spermatogenous tissue involves the three uppermost tiers of cells of the young antheridium.

5. In the development of the archegonium four neck canal cells are formed before the ventral canal cell and egg are differentiated. Some or all of the neck canal cells may later become binucleate.

6. A filamentous embryo consisting of four superimposed cells is formed before the appearance of vertical walls.

7. After the first transverse wall has appeared in the fertilized egg, both segments undergo transverse division, that of the hypobasal segment occurring first.

8. Of the two cells derived from the hypobasal segment, the lower one eventually gives rise to the foot and the upper one to the seta, while the two cells derived from the epibasal segment form the capsule.

9. The early embryogeny of *Cryptomitrium* closely resembles that of *Reboulia*, *Asterella*, and *Plagiochasma*.

10. An elater in *Cryptomitrium* is homologous with a single spore mother cell, not with a row of them.

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SPINDLE ABNORMALITIES IN MENTHA[1]

C. P. SWANSON AND RAY NELSON

(WITH TWENTY FIGURES)

Introduction

Various environmental agents (**9, 13, 23**) and meiotic studies of hybrids and mutants (**3, 6, 22**) have shown that the phenomenon of cell division, whether mitotic or meiotic, is a highly complex and delicately balanced one, a composite of several separate but integrated parts. That the study of these parts, made possible through a differential breakdown of the system, is far from complete, however, is evident from SCHRADER's recent review of mitosis (**17**). No theory yet proposed adequately covers all the phenomena observed. The present study deals with a genetically controlled breakdown in the development and behavior of the spindle in the genus *Mentha*.

The material employed was derived from a group of species and varieties of *Mentha* used in a disease-resistance breeding program. The plants consisted of clonal lines of the peppermint, *Mentha piperita* L. var. Mitcham, the spearmint, *M. spicata* L. (Huds.) var. *crispata*, together with six of their interspecific hybrids. Scarcity of meiotic material permitted a detailed study of only the parental species and two of the hybrids, although small samples of meiotic cells were obtained from all. No mitotic cells were studied. The spindle stained well with acetocarmine; thus it was possible to study accurately the form and structure of this portion of the division figure.

[1] Journal article no. 590, new series; Michigan Agricultural Experiment Station.

Observations

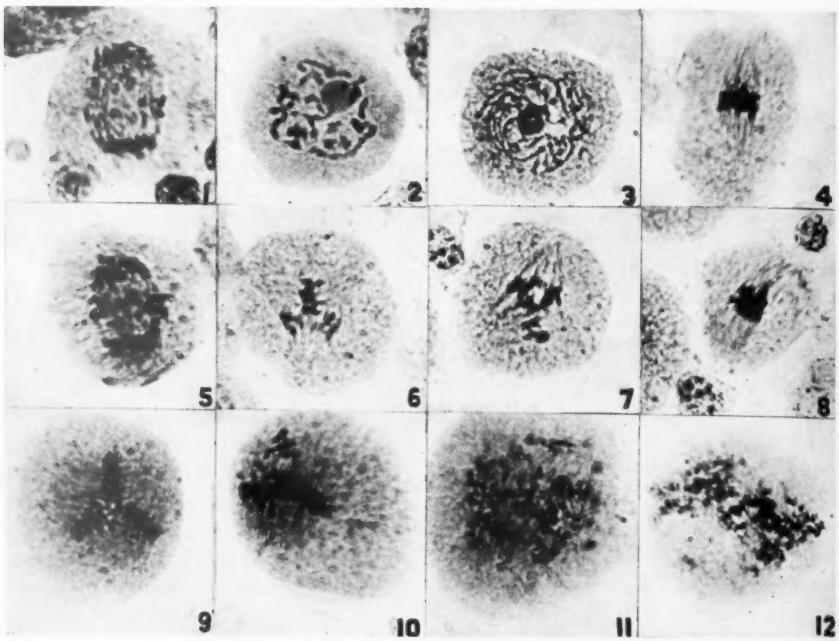
MEIOSIS IN GENERAL

Mentha piperita var. Mitcham is generally considered an interspecific hybrid, its suspected parents being *M. spicata* and *M. aquatica*. Its meiotic behavior is indicative of its hybrid origin. Almost completely pollen sterile, it sets little or no seed when selfed, even under the most favorable conditions. In crosses with *M. spicata* var. *crispata* some seed is produced, indicating that an occasional embryo develops. RUTTLE (**12**) has reported the diploid chromosome number as 65–69. The inability to distinguish between univalents and small bivalents, despite clear diakinetic figures, prevented accurate counts, although the number must be approximately 70. Considerable homologous pairing was evident, metaphase figures showing many bivalents. Pachytene stages showed unpaired threads and what appeared to be inversion loops. Diplotene and diakinesis stages were clear and well defined. Univalents were seen at metaphase, and at anaphase both lagging chromosomes and chromatin bridges were frequent (fig. 1). No spindle abnormalities were found, despite its considerable structural hybridity.

M. spicata var. *crispata* revealed no prophase irregularities of any kind. Pachytene stages showed numerous distinct chromatic knobs along the paired chromosomes (fig. 3). Diplotene, metaphase, and anaphase configurations (figs. 2, 4, 5), with the exception of spindle ab-

normalities, were regular, differing from SCHURHOFF'S (19) observations which led him to conclude that this species is a hybrid. It is probable that the plant investigated by SCHURHOFF was a genetically distinct strain, since other known strains of this species are highly sterile. Diplotene stages revealed the beginnings of major coil formation (fig. 2), and the coiling cycle followed the *Tradescantia* type of behavior (20). Normal metaphases presented a compact, distinctly outlined spindle, but 50 per cent of the cells revealed a breakdown of the regular spindle mechanism. This breakdown was expressed in varying degrees and seemed to take no particular direction. It might be supposed that this would lead to considerable pollen abortion, but 85 per cent of the pollen appeared viable.

The *piperita* × *spicata* hybrids, six in number, were produced by encircling a bed of *M. piperita* with plants of *M. spicata* var. *crispata* and allowing bees to effect pollination. Seeds were taken only from *M. piperita*. That the seedlings are true hybrids is evident from their intermediate morphology. In oil properties, however, the peppermint characteristics strongly dominate. In the pollen mother cells, the hybrids show the chromosome irregularities of *M. piperita* (fig. 5) and the spindle abnormalities of *M. spicata*. All the hybrids revealed evidences of



FIGS. 1–12.—Fig. 1, *M. piperita* var. Mitcham; anaphase. Fig. 2, *M. spicata* var. *crispata;* diplotene. Fig. 3, same; pachytene. Fig. 4, same; metaphase. Fig. 5, *piperita* × *spicata* hybrid; anaphase. Figs. 6, 7, *M. spicata;* tripolar spindles. Fig. 8, same; curved open spindle. Fig. 9, *piperita* × *spicata* hybrid; tripolar spindle. Fig. 10, same; metaphase-anaphase. Fig. 11, same; abortive spindle. Fig. 12, same; small abortive spindle.

spindle breakdown, but the degree of expression varied from plant to plant.

Very little functional pollen was produced by *M. piperita* or its hybrids. RUTTLE (12) has stated that the whole genus is characterized by high pollen abortion. Pollen degeneration begins shortly after the quartet stage, and only rarely was an anther sufficiently full of viable pollen properly to dehisce.

GENETIC CONTROL OF SPINDLE DEVELOPMENT

Several instances of genetic control of spindle development and behavior have been reported. BEADLE (1) found in maize a recessive gene for producing supernumerary mitoses following meiosis; CLARK (3) has shown, again in maize, that the gene *dv*, when homozygous, produces a divergent spindle in the meiotic and gametophytic divisions; DARLINGTON and THOMAS (6) have described a *Festuca-Lolium* hybrid with aberrant spindles; and UPCOTT (21) has shown that pollen sterility in *Lathyrus* is due to a recessive gene which produces a timing upset in spindle development. In animals, WALD (22) has reported the abnormal effect of the mutant gene *claret* in *Drosophila simulans* on maturation and cleavage divisions.

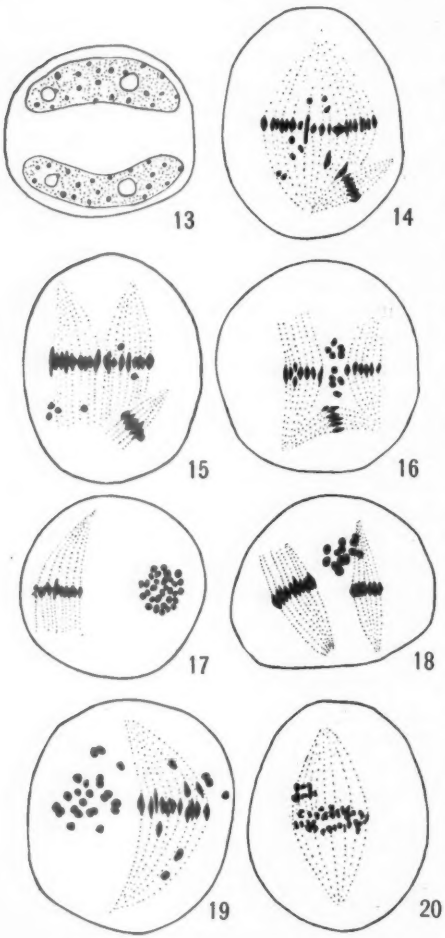
In the present study, the exact genetic control is still to be worked out. The data, however, indicate that the effect is a partially dominant one, since it has been transferred from a stable species, *M. spicata*, to all its hybrid offspring. It is known that the hybrid genotype is less well protected than the so-called "good" species against environmental changes; but that the spindle aberrancies are due to a definite genic control and not to hybridity or to a general gene unbalance is indicated by the derivation of the effect from the more regular of the two parents in meiosis, and by the fact that the hybrids show a lower percentage of abnormal cells than did *M. spicata*. The chromosomal unbalance contributed by *M. piperita* had no additive effect, in so far as the spindle is concerned. The genic control is not dominant in the sense that the effect is shown in every cell, however, but the degree of expression, or penetrance, of the presumably heterozygous condition in the hybrids is less than that of the original species, *M. spicata*. A varying percentage of the cells show spindle abnormalities, and these very irregularly. Within the same bud some anthers are completely normal, while others are equally abnormal; usually the anthers contain a mixture of normal and abnormal cells. Environmental conditions, both internal and external, appear to be the deciding factors, and since—as will be shown later—the spindle abnormalities take no specific form, what appears to be transmitted from *M. spicata* to its hybrid offspring is a susceptibility to environmental changes expressed through an instability of spindle form. There appears to be no directive control of spindle shape and behavior such as CLARK (3) has shown in the case of the divergent spindles in maize.

STRUCTURAL AND TIME VARIATIONS IN SPINDLE DEVELOPMENT

Variation in spindle development can be shown in two ways: (*a*) a structural variation expressing itself through deviations in the polar conditions as well as in spindle form and number; (*b*) a time variation expressing itself through lack of co-ordination with other nuclear processes, notably the chromosome spiralization cycle.

Mentha spicata, the male parent, showed the greatest percentage of cells with spindle upsets, but the range of vari-

ation was narrow. Only tripolar (figs. 6, 7) and curved open spindles (fig. 8) were found. The extra pole was either equal in size and structure to the other two (fig. 6), or it was small and abortive (fig. 7), merely distorting the spindle.



FIGS. 13–20.—Figs. 13–16, *piperita* × *spicata* hybrids; complete chromosome complement not shown (13, interphase; 14, accessory spindle at metaphase I; 15, 16, accessory spindles with central spindle divided). Figs. 17–20, *piperita* × *spicata* hybrids; complete chromosome complement not shown (17, metaphase II with curved open spindle; 18, metaphase I with two independent spindles; 19, metaphase I, curved spindle; 20, early anaphase).

It would be expected that tripolar spindles would lead to the formation of three nuclei at anaphase, as DARLINGTON and THOMAS (**6**) have shown; but such has not been found, although many cells were examined with this in mind. That no more than two nuclei are formed is probably due to the size and shape of the interphase nucleus (fig. 13), the long kidney-shaped structure occupying nearly the whole of one hemisphere of the cell, with its ends closely approximating those of its sister nucleus. Any extra nuclei which might be formed as a result of a tripolar condition would be incorporated into the larger nucleus, which, so far as the present observations show, forms as a rounded nucleus at telophase and then enlarges and spreads laterally, following the curvature of the cell walls.

The curved spindles were of the open type (fig. 8). They appeared to be too narrow and too long to be accommodated within the confines of the cell; hence they bend with the cell wall. The chromosomes seem to have no difficulty in reaching the indefinite polar regions to form a single regular nucleus. In this respect the spindles were similar to the divergent spindles in maize (**3**), except that only single telophase nuclei were formed. DOBZHANSKY (**7**) has described similarly curved spindles in *Drosophila*, but which possessed clearly differentiated polar regions.

The clonal line of *M. spicata* used in this study is a highly fertile one, in contrast to nearly all the varieties and species of this genus (**12**). Apparently, therefore (with the possible exception of some tripolar spindles), the abnormalities of the division mechanism lie within the range of diversity within which the spindle must operate to give rise to regu-

lar anaphase segregation and telophase reorganization. No timing irregularities were found, and the second division was regular in all respects.

The range of structural variation was markedly increased in the hybrids, although the percentage of abnormal cells was decreased. Tripolar spindles were frequent (fig. 9), as were curved and open types (fig. 19). Even more striking were the cells with multiple spindles. The spindles in these cells were generally united with each other at the polar regions (figs. 14–16), the main spindle being either entire (fig. 14) or split into two parts (figs. 15, 16). As a rule the accessory spindles were small, always located adjacent to, or connected with, one of the major poles and possessed of either open or closed poles, or both. Only infrequently were two separate and distinct spindles formed at metaphase I (fig. 18). This figure appears similar to one in metaphase II, but the bivalents lying free in the cytoplasm show it to be otherwise.

Second division abnormalities were comparatively rare, only one (fig. 17) being found among several hundred cells.

Figure 10 is of particular interest. The spindle is somewhat curved, with the indistinct polar regions displaced to the left of the middle of the equatorial plate. The figure superficially resembles that of an unequal tripolar condition, but careful examination of this and a number of other similar metaphase configurations revealed no spindle material associated with the "third pole." Likewise the orientation of the bivalents shows no influence of a tripolar condition. Apparently the bivalents at the right side of the plate have moved into anaphase before the members situated on the left side, these still being in a metaphase state. It might be supposed that two closely approximated spindles are present, with one entering anaphase before the other; but this seems not to be the case, for even in cells with two spindles widely separated (fig. 18) the division is always exactly synchronized. In cells of this sort the spindle was always located on one side, with the equatorial plate closely abutting the cell wall. The only apparent conclusion from these anomalous metaphase-anaphase figures is that the proximity of the equatorial plate to the cell wall has upset the usual synchronization of anaphase segregation and has allowed some of the bivalents to enter anaphase before the others. If this interpretation be the true one, and the observations seem to allow of no other, it serves as evidence for BELAR'S (2) hypothesis of chromosome autonomy at the time of initial separation. If a polar force were in evidence at this time, it is difficult to envision a portion of the chromosomes moving into anaphase before the others. SAX and O'MARA (14) likewise favor chromosome autonomy, particularly where the cramped conditions of the pollen tube do not permit the functioning of a normal spindle.

Variations in the timing relationships of the spindle, in so far as they relate to the chromosome spiralization cycle, have also been found in the *piperita* × *spicata* hybrids. Development of the spindle may be precocious or delayed. When precocious, the chromosomes do not stain deeply, and the nucleolus may still be present on the metaphase plate. More often the spindle was delayed in its development. The chromosomes appear indistinctly stained, as when the spindle is precocious; but the absence of the nucleolus and the despiralized condition of the chromosomes indicate that the chromosomes are in late anaphase or early telophase, as far as their coiling is concerned (cf. figs. 11, 12 with 4). In figure

11 the spindle has failed to develop sufficiently to orient the bivalents, many of which are in a despiralized and desynapsed condition. Delayed spindles tended to be undersized (fig. 12), permitting the congression and orientation of only a limited number of bivalents on to a narrow equatorial plate lying between two masses of unoriented chromosomes.

Figure 20 presents an anomalous situation. The staining of the spindle permitted no other interpretation than that two equatorial plates were associated with but a single spindle, with the smaller upper plate lying above and to one side of the larger lower plate. Both groups of chromosomes are in early anaphase. The state of spiralization of the chromosomes indicated that the spindle was somewhat delayed. This situation is difficult to evaluate in the light of modern cell mechanics, and since it was the only cell of its kind found in this study, it seems best merely to record it until further study is made.

Discussion

This and other studies (3, 4, 6, 8) permit a more critical analysis of the origin, development, and behavior of the spindle, a structure whose function has been clearly elucidated but about which comparatively little else is known with certainty.

To understand and interpret the abnormal spindle development in *Mentha*, it is necessary to consider the origin and behavior of the normal spindle. The studies of BELAR (2), SCHRADER (16), HUGHES-SCHRADER (10), and FANKHAUSER (8) have been clarifying. They have shown that the spindle is probably a composite structure in most organisms. SCHRADER and HUGHES-SCHRADER have demonstrated in the coccids, where the centrosomes are lacking, that the spindle is a truncate, divergent affair arising from the development of the chromosome and its matrix; on the other hand, FANKHAUSER has shown that the spindle can be initiated in enucleated egg fragments, hence quite apart from the chromosomes (also the "unattached" spindles of DARLINGTON and THOMAS, 6). A normal spindle, therefore, at least of higher plants, appears to be the product of the chromosomes plus the pole determinants, co-operating with each other and synchronized in time and space, although either can produce the spindle unaided in some organisms. These are the centrosome and centromere spindles of DARLINGTON (5). The behavior of the divergent spindle (3) would seem to indicate that the centrosome portion can be dispensed with, in so far as anaphase movement is concerned, although more or less necessary for complete telophase reorganization of the nucleus.

The structural abnormalities described here seem best interpreted as variations in the behavior of the pole determinants. The centromere (chromosome?) portion of the spindle is usually present; only rarely is there complete breakdown of the spindle. Extra pole determinants, which probably arise *de novo* rather than from some invisible but permanent structure, lead to multipolar or to multiple spindles; a diffuseness at the poles, due either to the absence of a determinant or to its weak coalescent properties, leads to a divergent spindle whose structure is largely the result of chromosomal activity. OSTERHOUT (WILSON, 23) has shown in the microsporocytes of *Equisetum* that a multipolar condition regularly precedes a bipolar spindle, but it is doubtful whether such a state exists in *Mentha*.

The timing abnormalities reveal that the spindle is smaller in size the more it is delayed in time (fig. 12). This must mean that as the chromosomes begin to despiralize the centromeres are unable to

function to their full extent, and congression, orientation, and spindle formation are incomplete. This can be demonstrated in other plants. UPCOTT (21) has shown that a gene in *Lathyrus odoratus* upsets the spindle-chromosome co-ordination by delaying the spindle, while SWANSON (20) has reported that a precocious or delayed spindle can be readily induced at meiosis in *Tradescantia* by heat treatments.

Of interest from the point of view of cell mechanics are the accessory spindles (figs. 14–16). They are formed by small groups of chromosomes which had failed to be included in the major spindle. Yet apparently similar groups of free chromosomes failed to produce similar spindles (figs. 18, 19). WALD (22) has shown that the initiation of spindle formation in *Drosophila simulans* is dependent upon the presence of the II and III chromosomes. While specific chromosomes cannot be identified in *Mentha*, it is possible that the presence and activity of certain chromosomes is necessary for spindle initiation. In the case of *D. simulans*, and possibly in *Mentha*, it appears that the control of spindle-forming activity has been transferred from the individual chromosomes, as in the coccids (15), to specific chromosomes—an instance of genetic specialization.

KOLLER (11) and DARLINGTON (5) postulate polar repulsions as important in nuclear behavior. Evidence for this is largely inferential; recent data are against it. CLARK'S (3) work on divergent spindles, SCHRADER'S (18) and SAX and O'MARA'S (14) on curved spindles, DARLINGTON and THOMAS' (6) on twisted spindles, and the present study all challenge polar repulsions. Effective polar repulsions should not only bring about correct orientation of the chromosomes but should invariably produce a straight and symmetrical spindle. Such is not the case. Also equatorial orientation of the chromosomes can occur when no regular polar regions exist, implying that the agents of orientation are probably the chromosomes themselves. The autonomy of the chromosomes in initiating anaphase separation has been discussed in connection with figure 10. BELAR'S (2) theory of later anaphase movement, based on growth of the "stembody," is not supported by observations in *Mentha*, for no narrowing of the spindle in late anaphase is apparent (figs. 1, 5). CLARK (3) has shown this to be true also in maize. That the chromosomes are not alone responsible for anaphase movement is indicated by a study of cells with two spindles (fig. 18). When in anaphase, the movement of the chromosomes is always synchronized. A certain cytoplasmic condition must be necessary before the chromosomes express their autonomy in anaphase separation. The synchronous division of many adjacent cells in meiosis, both in plants and animals, bears this out.

Summary

Meiotic spindle irregularities in *Mentha piperita*, *M. spicata*, and six of their interspecific hybrids were investigated, including both timing and structural variation in spindle development and behavior. These spindle abnormalities are dependent upon the action of a gene transmitted from *M. spicata* to all its offspring. The effect of the gene is to induce an unstableness of spindle form and behavior which is subject to environmental differences. The gene exerts no directive influence on the spindle change, for irregularities were of many kinds.

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COMPARATIVE ACTIVITY OF SYNTHETIC AUXINS AND DERIVATIVES

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Introduction

The object of the present study was to determine the comparative activity of both known and new synthetic auxins and derivatives, applied to intact and decapitated *Avena* test plants in two carriers, agar and lanolin. The technique which employs the *Avena* coleoptile is considered by many a preferred method for the quantitative determination of auxin activity. KÖGL and KOSTERMANS (7) with this method, and KOEPFLI (6) with the pea test, found that compounds which promote growth must, among other requirements, possess a free carboxyl group, or—if esterified—be readily hydrolyzable; their tests all involved the use of agar or aqueous solution as a carrier.

When the bending response of tomato leaves and stems was used as a criterion of auxin activity, using lanolin as a carrier, ZIMMERMAN and HITCHCOCK (12, 13) found that the methyl esters of indoleacetic and indolebutyric acids were more than twice as active as the free acids; methyl naphthaleneacetate was half as active as naphthaleneacetic acid.

METHODS.—In the course of this work, various synthetic auxins, and the amides and esters of some of them, have been investigated by the deseeded test method of SKOOG (see 1). To determine the influence of the carrier on the results obtained, lanolin as well as the usual agar blocks was applied to decapitated coleoptiles. Such lanolin blocks were prepared by pouring warm lanolin containing the various substances, in different concentrations, into standard-volume brass molds standing directly upon ice cakes. The blocks cut from these were of the same volume as the agar ones (10 mm.^{3}) and were applied to decapitated coleoptiles in the same manner that the agar blocks were applied.

In tests involving the use of lanolin on intact coleoptiles, the auxins and their derivatives were mixed in anhydrous lanolin and applied unilaterally to the distal 5 mm. of intact *Avena* coleoptiles (on the narrow side of the coleoptile, parallel with the underlying vascular bundle). In those experiments where agar blocks were applied to the side of intact coleoptiles, they were made to adhere by first applying a small drop of 5 per cent gelatin.

Avena plants (variety Victory) were grown in the usual water cultures. All were deseeded, and the same conditions of temperature and humidity (25° C. and 85–90 per cent) were maintained during the application period for both lanolin and agar tests. Conclusions as to activity were based on shadowgraphs of test plants taken after 5 hours.

Experimental results

APPLICATION IN AGAR TO DECAPITATED COLEOPTILES

Potassium salts of indoleacetic, indolebutyric, and naphthaleneacetic acids are as active as free acids (table 1). This is in accord with previous results ob-

tained by the *Avena* assay (4, 11, 2) and by the tomato-bending response (13).

to be as active as the free acid. Ethyl indoleacetate is only one-third as active

TABLE 1

RELATIVE ACTIVITY OF SYNTHETIC AUXINS, DETERMINED BY DESEEDED AVENA TECHNIQUE, USING AGAR AS THE CARRIER (ON DECAPITATED COLEOPTILES). AT LEAST 12 TEST PLANTS WERE USED PER DILUTION ASSAYED, AND ALL CURVATURES ARE IN THE PROPORTIONALITY RANGE. IN MOST INSTANCES, CALCULATIONS OF RELATIVE ACTIVITY IN COMPARISON WITH INDOLEACETIC ACID ARE BASED ON CURVATURES OBTAINED WITH THE LOWER CONCENTRATIONS

COMPOUND	SOURCE*	CONCENTRATION (MG.) PER LITER OF AGAR MIXTURE; AVENA CURVATURE IN DEGREES				RELATIVE EFFECTIVENESS (INDOLEACETIC ACID = 100)
		CONC.	CURV.	CONC.	CURV.	
Indoleacetic acid	VIII	0.010	10.7‡	0.020	19.6‡	100
Potassium indoleacetate	III	0.010	10.1	0.020	21.5	94
Methyl indoleacetate	II	0.010	6.6	0.020	12.6‡	62
	III	0.010	6.5†	0.020	13.8†	61
Ethyl indoleacetate	II	0.020	6.2‡	0.040	10.5†	29
	IX	0.020	6.0†	0.040	10.8†	28
Indoleacetamide§	IV	0.500	0	2.0	0	0
	V	0.500	0	1.0	3.7†	0.4
Indolebutyric acid	III	0.100	4.1	0.200	8.7	4
	VIII	0.160	8.3	0.320	17.0	5
Potassium indolebutyrate	III	0.200	8.1†	0.400	16.5	4
Methyl indolebutyrate	I	0.160	8.1	0.320	17.5	5
	II	0.160	8.0	0.080	4.9	5
	III	0.160	6.9†	0.320	14.1	4
Ethyl indolebutyrate	II	0.160	8.1	0.320	12.0†	5
Naphthaleneacetic acid (all derivatives are alpha form)	IV	0.050	10.1	0.100	19.8	19
	III	0.050	10.3	0.100	18.7†	19
Potassium naphthaleneacetate	III	0.050	8.6	0.100	18.8	16
Methyl naphthaleneacetate	II	2.56	2.8	5.12	4.7	0.09
	III	2.56	2.1†	5.12	3.3†	0.06
	VI	2.56	0†	5.12	1.7†	0.03
Ethyl naphthaleneacetate	I	2.56	0	5.12	0	0
Naphthaleneacetamide§	IV	2.5	0†	5.0	0	0
	VI	2.56	0†	5.12	2.9	0.05
	VII	2.56	0	5.12	4.8	0.09
Naphthoxyacetic acid (all derivatives are beta form)	IX	0.2	0	5.0	0	0
Ethyl naphthoxyacetate	IX	0.2	0	5.0	0	0
Naphthoxypropionic acid	IX	0.2	0	5.0	0	0
Dichlorophenoxyacetic acid	IV	5.0	6.7	10.0	10	0.1

* Source of compounds: I–III, from P. W. Zimmerman, Boyce Thompson Institute for Plant Research (I received 1936; II and III received in February and July, 1941). IV, Dow Chemical Company. V, K. V. Thimann, Harvard University. VI, American Chemical Paint Company. VII, R. H. Manske, National Research Council of Canada. VIII, Merck and Company. IX, P. W. Zimmerman (received March, 1942).

† Average of 24 test plants.

‡ Average of 36–60 test plants.

§ Upon hydrolysis with 1N NaOH, indoleacetamide and naphthaleneacetamide gave solutions which, after neutralizing, had at least half the auxin activity which could be expected from a theoretical yield of free acids.

In general, the esters are less active than the corresponding free acids, with the exception of the methyl and ethyl esters of indolebutyric acid; these appear as the free acid; and the methyl and ethyl esters of naphthaleneacetic acid are almost completely inactive. These results are in general similar to those re-

ported elsewhere for ester derivatives; that is, esters of certain active acids are active, while others are inactive. Indoleacetamide and naphthaleneacetamide are almost completely inactive. β-naphthoxyacetic acid, its ethyl ester, and β-naphthoxypropionic acid are also completely inactive.

The response of *Avena* to esters of naphthaleneacetic and naphthoxyacetic acids, and the amides of indoleacetic and naphthaleneacetic acids, has not been reported on heretofore. Dichlorophenoxyacetic acid, recently shown to be active in inducing parthenocarpy and leaf-form changes (**15**), shows but slight activity in the *Avena* test.

Application in agar to intact coleoptiles

No curvatures were obtained with indoleacetic, indolebutyric, and naphthaleneacetic acids when applied in agar to intact coleoptiles. All were tested at concentrations of 0.5, 1.0, and 5.0 mg. per liter; in addition, indoleacetic acid was tested at 10, 20, and 40 μgm. per liter.

Laibach and Kornmann (**8**) applied agar blocks containing auxin from pollen of several different species of orchids at different levels on intact *Avena* test coleoptiles. Those applied near the tip gave very small curvatures, whereas those applied below the tip—at one-third to one-half the length of the coleoptile—induced pronounced curvatures within 5 hours. Following their technique as closely as possible, and using the preceding concentrations of the synthetic substances, no *Avena* curvatures were obtained. Extracts of hickory pollen, which by assay on decapitated coleoptiles give 4.6–9.6 million TDC per gram (equivalent to 46–96 μgm. indoleacetic acid per gram), also give no curvatures when applied in agar blocks to intact coleoptiles. The concentration used (1:100) approximates that employed by Laibach and Kornmann.

Application in lanolin blocks to decapitated coleoptiles

When lanolin is the carrier (table 2), concentrations of the acids required to produce *Avena* curvatures close to 10° are approximately ten to fifty times as great as for agar. The activity ratios of indoleacetic, indolebutyric, and naphthaleneacetic acids, when applied in lanolin, are 100:11:11, respectively; whereas in agar the ratios are 100:5:19. The response to indoleacetic acid in each of the two carriers is arbitrarily set at 100.

TABLE 2

Relative activity of synthetic auxins as determined by deseeded Avena technique, but employing blocks of lanolin rather than of agar

Compound	No. of test plants	Concentration per liter of lanolin (mg.)	Avena curvature (°)	Relative effectiveness (indoleacetic acid = 100)
Indoleacetic acid	24	0.3*	8.0	100
Indoleacetic acid	24	0.6	11.9	.....
Indolebutyric acid	24	2.5*	7.5	11
Indolebutyric acid	24	5.0	7.5	.....
Naphthaleneacetic acid	12	2.5*	7.4	11
Naphthaleneacetic acid	24	5.0	11.2	.....

* Still lower concentrations give curvatures too inconsistent to be relied upon; these may therefore be considered threshold values.

The lack of proportionality curvatures (table 2) is to be noted.

Application in lanolin to intact coleoptiles

The data from lanolin tests on whole coleoptiles are presented in table 3. The

comparative activities of the several derivatives in relation to their corresponding acids are not greatly different, whether applied in agar or in lanolin. Methyl and ethyl indoleacetates are the outstanding exceptions: the former is one and one-half times as active as the acid, the latter only about one-twentieth as active; the ethyl ester of indolebutyric is only one-half as active as the free acid.

Indoleacetamide, when applied in lanolin to intact coleoptiles, gives a threshold response at 1500 mg. per liter of lanolin, only one five-thousandths as active as the free acid. Naphthaleneacetamide does not give any response when tested at concentrations ranging from 5 to 5000 mg. per liter of lanolin. Hence, in the *Avena* test the amides are essentially inactive, whether applied in agar or in lanolin, to decapitated or intact coleoptiles.

Threshold concentrations in lanolin are the same, whether the coleoptiles are intact or decapitated, in spite of the fact that the area of application to the test plants differs.

TABLE 3

RELATIVE ACTIVITY OF SYNTHETIC AUXINS, DETERMINED BY UNILATERAL APPLICATION IN ANHYDROUS LANOLIN TO WHOLE AVENA COLEOPTILES (DESEEDED PLANTS). AT LEAST 12 TEST PLANTS USED PER DILUTION ASSAYED. CURVATURES REPRESENT THRESHOLD RESPONSES (LOWEST CONCENTRATIONS GIVING CURVATURES OF 5–10 DEGREES)

COMPOUND	SOURCE*	CONCENTRATION PER LITER LANOLIN REQUIRED TO GIVE THRESHOLD RESPONSE (MG.)	CURVATURES (°)	RELATIVE EFFECTIVENESS (INDOLEACETIC ACID = 100)
Indoleacetic acid	VIII	0.3	9.0	100
Methyl indoleacetate	II	0.2	9.1	150
Ethyl indoleacetate	II	5.0†	6.6	6
Indoleacetamide	IV	1500.0	14.8	0.03
Indolebutyric acid	VIII	2.5	6.6	12
Methyl indolebutyrate	II	2.5	5.6	12
Ethyl indolebutyrate	II	5.0	6.8	6
Naphthaleneacetic acid	IV	2.5	6.7	12
Methyl naphthaleneacetate	VI	No response‡	0	0
Naphthaleneacetamide	IV	No response§	0	0
Naphthoxyacetic acid	IX	No response‖	0	0
Ethyl naphthoxyacetate	IX	No response‖	0	0
Naphthoxypropionic acid	IX	No response‖	0	0
Dichlorophenoxyacetic acid	IV	80.0	3.8	0.2

* Sources as for table 1.

† Gave very inconsistent results in four sets of tests on three batches of materials. Value reported is probably maximum.

‡ No curvature in six sets of tests at concentrations of 5–80 mg. per liter of lanolin.

§ No curvature in three sets of tests at 5–160 mg. per liter of lanolin.

‖ No curvature in two sets of tests at 2.5–10 mg. per liter of lanolin.

LEVULINIC ACID

Reports finding their way into the trade literature (5) regarding the activity of levulinic acid make it of interest to assay this substance in the usual *Avena* test. No activity is obtained at concentrations of 0.1, 0.2, 1.0, and 5.0 mg. per liter of agar mixture. Negative results are obtained also when it is tested in lanolin on intact coleoptiles at concentrations of 2.5, 5.0, and 10.0 mg. per liter of

lanolin. To determine whether it might possibly increase the activity of an auxin such as indoleacetic acid, levulinic acid in concentrations of 0.2 and 1.0 mg. per liter was made up in 1.5 per cent agar with indoleacetic acid (the latter at a 0.010 mg. per liter concentration). The lower concentration of levulinic acid, with the indoleacetic, gave *Avena* curvatures of 10.7°; the higher concentration, 11.0°. Indoleacetic-acid controls tested at the same time gave curvatures of 11.7°. Earlier tests on levulinic acid in straight growth of the *Avena* coleoptile (2) were also negative. Thus this compound induces no response in the *Avena* test, nor does its presence accelerate the response of the coleoptile to indoleacetic acid.

Discussion

It has been found that (*a*) when applied in agar to decapitated *Avena* plants, certain auxins as free acids are more active than ester or amide derivatives, which agrees with and extends data already in the literature; (*b*) when applied in lanolin to whole (intact) *Avena* coleoptiles, one ester is more active than the corresponding free acid, which only partly confirms data in the literature (in which tomato was used as the test plant). The hypothesis that the ester and amide derivatives must first be hydrolyzed before they are active still cannot be answered directly. In agar, both the methyl and ethyl esters of indolebutyric acid are apparently hydrolyzed readily; with indoleacetic acid, only the methyl ester; with naphthaleneacetic acid, apparently neither ester is hydrolyzed. In lanolin the exceptional activity of methyl indoleacetate might be interpreted as indicating that hydrolysis need not occur; there is insufficient evidence to eliminate other possibilities.

The data indicate that when the same test plant is used (*Avena*), the relative activities of auxins differ according to the nature of the carrier in which they are applied. Ten to fifty times the concentration of different growth substances is required for threshold responses when applied in lanolin (whether blocks or unilateral smears) as compared with agar, indicating that the rate of penetration of auxins from lanolin into decapitated *Avena* test coleoptiles is markedly slower than that from agar. Against this is the fact that the substances applied in agar to intact coleoptiles induced no response at all. This latter point can only mean that when the epidermis is intact, synthetic growth substances in agar penetrate into the underlying tissue so slowly that no response is induced in the 5-hour test period. The extent to which the agar makes a poor contact with the coleoptile tissue is not known, and this also may be an important factor in the failure of agar as a carrier on intact coleoptiles. LAIBACH and KORNMANN'S positive results were obtained with orchid pollen extracts. SNOW (10) found that extracts of pea shoots gave both positive and negative curvatures, when applied in strips of blotting paper to intact *Avena* coleoptiles.

MITCHELL and STEWART (9) report that naphthaleneacetamide is inactive in agar, in the *Avena* test (0.25 mg. per liter of agar), and also when unilaterally applied to stems of tomato plants at 2 per cent in lanolin. However, they obtained *Avena* curvatures when naphthaleneacetamide was applied unilaterally at 172 mg. per liter of lanolin (0.02 per cent).

Different species differ in degree of response to a given compound in a given carrier; tomatoes and *Avena*, for example, do not have the same threshold re-

sponses to different synthetic auxins applied in lanolin (table 4). Such data are not strictly comparable, because the tomatoes were greenhouse grown, and temperatures doubtless fluctuated considerably. It is clear, however, that for the active compounds it requires eight to eighty times the concentration in lanolin to induce the tomato response as compared with the *Avena* response. Certain compounds, such as naphthoxyacetic acid and methyl naphthaleneacetate, are completely inactive on *Avena* but are very active in inducing epinasty and other responses in tomato (**12, 14**).

It would seem that, given the same carrier (lanolin), the response of different test plants depends in part upon their respective growth rates and in part upon their species. In the case of *Avena* vs. tomato, it is reasonable to expect that etiolated coleoptiles of *Avena* grow more rapidly than greenhouse-grown tomato plants; hence the necessity of higher concentrations of growth substances to induce the response in tomato. However, some compounds are active on tomato which are completely inactive on *Avena*. Given a different carrier (agar vs. lanolin) and the same kind of test plant (such as *Avena*), the response depends upon the rate of penetration from the carrier.

TABLE 4

RELATIVE ACTIVITY OF SYNTHETIC AUXINS, DETERMINED BY UNILATERAL APPLICATION IN ANHYDROUS LANOLIN TO WHOLE AVENA COLEOPTILES AND TOMATO PETIOLES (ZIMMERMAN). AT LEAST 12 TEST PLANTS USED PER DILUTION ASSAYED. ONE LITER OF LANOLIN = 860 GM.

COMPOUND	SOURCE	THRESHOLD CONCENTRATION REQUIRED TO GIVE DEFINITE CURVATURES AFTER 5 HOURS, MG./L. OF LANOLIN (FROM TABLE 3)	THRESHOLD VALUES, % IN LANOLIN		
			AVENA COLEOPTILE (FROM TABLE 3)	TOMATO PETIOLE (ZIMMERMAN ET AL.)	
				1936	1937
Indoleacetic acid	VIII	0.3	0.000035	0.00125	0.0001
Methyl indoleacetate	II	0.2	0.000023	0.0006	0.0001*
Ethyl indoleacetate	II	5.0†	0.00058	.....	0.0001*
Indoleacetamide	IV	1500	0.17	.....	No response*
Indolebutyric acid	VIII	2.5	0.00029	0.025	0.01
Methyl indolebutyrate	II	2.5	0.00029	0.01	.....
Ethyl indolebutyrate	II	5	0.00058	.....	.....
Naphthaleneacetic acid	IV	2.5	0.00029	0.0025	0.0001
Methyl naphthaleneacetate	VI	No response	.....	0.005	0.0001*
Naphthaleneacetamide	IV	No response	.....	.....	ca. 0.0001*
Naphthoxyacetic acid	IV	No response	.....	.....	0.005*

* Private communication. We are grateful to Dr. P. W. Zimmerman for certain samples, and for permission to include these data.
† After 10 hours there sometimes are threshold curvatures at 5 mg./l., but such results are irregular.

The preceding data emphasize still further (*a*) the conclusion of several investigators (**11, 13, 3**) that no one test plant or test method can suitably serve for determining the relative activities of various synthetic auxins, and (*b*) the importance of the carrier in determining relative activities, even when the same test plant is used.

Summary

The activity of indoleacetic, indolebutyric, naphthaleneacetic, dichlorophenoxyacetic, and naphthoxyacetic acids

and certain derivatives has been tested on deseeded *Avena* coleoptiles. Intact as well as the usual decapitated coleoptiles were employed, and both agar and lanolin blocks (and lanolin in unilateral smears) were used as carriers.

1. When tested in agar on decapitated coleoptiles, potassium salts of indoleacetic, indolebutyric, and naphthaleneacetic are as active as the free acids. Esters of indolebutyric are of about the same activity as the acid; esters of indoleacetic are less active than the corresponding acid; and esters of naphthaleneacetic acid are inactive. Indoleacetamide, naphthaleneacetamide, and dichlorophenoxyacetic acid are but slightly active. Naphthoxyacetic, its ethyl ester, and naphthoxypropionic acids are completely inactive (although reported by others to be active in the tomato test).

2. When applied in agar to intact coleoptiles, indoleacetic, indolebutyric, and naphthaleneacetic acids are completely inactive.

3. When blocks of lanolin rather than agar are applied to decapitated coleoptiles, ten to fifty times the concentration of indoleacetic, indolebutyric, and naphthaleneacetic acids must be used to give threshold curvatures. In agar, the relative activities of the acids are 100:5:19, respectively; in lanolin, 100:11:11.

4. When applied in lanolin to intact coleoptiles, indoleacetic, indolebutyric, and naphthaleneacetic acids have the same activity as when applied to decapitated coleoptiles; that is, threshold concentrations are the same whether the coleoptiles are intact or decapitated.

5. Levulinic acid is completely inactive in the *Avena* test, whether applied in lanolin or agar, and has no complementary effect when applied together with indoleacetic acid.

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ANATOMY AND HISTOLOGY OF THE EUREKA LEMON

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(WITH FORTY-SEVEN FIGURES)

Introduction

VOLKAMER (62) and RISSO and POITEAU (43) described and illustrated gross aspects of many species of *Citrus*. Their reports are of morphological significance mainly because they served as the background for the work of BONAVIA (9), who, without significant anatomical data of his own, presented an interpretation of the citrus fruit based upon comparative anatomy. He regarded the rind as one whorl of sterile carpels, while the pulp was considered a second whorl of carpels which were fertile and whose oil glands (juice sacs) protruded into the locules. Evidence for this concept of the juice sacs was found in the related genus *Oegle*, where more definite oil glands were found projecting from the pericarp into the locules. BONAVIA'S concept of the two whorls of carpels is similar to SAUNDERS' (45) concept of the nature of the angiosperm gynoecium.

Phases of the macroscopic structure of the fruits of several species of *Citrus* have been described (11, 12, 25, 51, 65). ROSS (44), in describing the lemon fruit, applied the classic concept of the carpel in interpreting the different vascular bundles of the gynoecium, although he did not follow their courses. He referred to the juice sacs as carpel hairs but presented no more histological data than had GREW (25) three centuries earlier.

References to teratological flowers and fruits of *Citrus* have been discussed by PENZIG (39) and MASTERS (32). Among these are the instances of extra whorls of carpels and the occurrence of sterile digitate carpels. The latter, in a variety of lemon, has been used by SAUNDERS (46) as evidence in favor of the theory of polymorphic carpels. In this variety only the dorsal part of each carpel elongates, while the marginal portion remains unseparated from the apex of the receptacle. Neither ovules nor locules are produced. This was interpreted as failure of the "fertile carpels" to develop while the "sterile carpels" do develop and form the anomolous structures.

PAYER (37) has presented the ontogeny of the floral parts of *C. aurantium*. PENZIG (38) has discussed histological data for the same species. He disagreed with PAYER and considered two whorls of stamens present rather than one. Also, his account of the courses of vascular bundles differs from that of VAN TIEGHEM (61) for the same species. Some histological data on *C. decumana* and *C. hystrix* were given by comparison. TSCHIRCH (58) described and figured some aspects of histology of the gynoecium and fruit of *C. vulgaris*. Both he and PENZIG (38) distinguished two kinds of carpellary emergences into the locule. The long ones, the juice sacs, were considered histologically different from, though similar in origin to, the short dome-shaped ones which had papillose cells and which TSCHIRCH thought secreted mucilaginous material into the locule. He described the formation of the oil glands. The structure of the rind has been discussed also by others

(8, 9, 14, 19, 20, 29, 38, 40) for various species.

BIERMAN (8) presented data on the cytoplasmic inclusions of the parenchymatous cells of the fruit of *C. vulgaris* and described seeds with several embryos in this and other species. OSAWA (35) dealt mainly with sporogenesis and embryogeny in *C. trifoliata* and gave some causes of the seedless condition in Washington Navel orange and in *C. nobilis*. Parthenocarpy (26), fertilization (13), and nucellar embryos (22, 23) have also been discussed. Recently data on blooming and fruit setting have been reported for several species (1, 42).

Several reports (33, 50, 51, 56) have appeared on the taxonomy and phylogeny of that section of the Rutaceae to which *Citrus* belongs. One of these (56) attempts to correlate the degree of fusion of the vascular traces, mainly those to perianth and stamens, with phyletic groups within the family and to make clear the nature of the stylar canals, interpreting the gynoecial disk as a vestigial stamen whorl. In some species definite correlation between location of oil glands and ends of bundles was found.

Material and methods

Collections were made of flowers and fruits of Eureka lemon of various ages at Lower Rio Grande Valley Experiment Station, Westlaco, Texas, by S. H. YARNELL in 1938 and by J. F. WOOD in 1941 and 1942. Certain stages were collected also at Riverside, California, by Dr. H. E. HAYWARD in 1941. The material was killed in various solutions, and serial sections were prepared by the paraffin technique. Young inflorescenses, flowers, and fruits up to 15 mm. in diameter were imbedded and sectioned whole, while only suitable pieces of larger fruits were used. Considerable difficulty arose from the presence of quantities of hesperidin raphides throughout the inner parts of fruits of certain ages. No satisfactory way of removing them was found.

Observations

PEDICEL

Cymose flowers arise in axils of the leaves. Pedicels are very short and are subtended by single minute bracts. Many of the lateral flowers contain well-developed stamens but abortive pistils. These and other flowers which do not set fruit abscise at the base of the pedicel, although the petals, stamens, and pistils sometimes abscise first.

The cortex is composed of isodiametric cells and abundant air spaces. Oil glands occur frequently in the outer part of the cortex. Like those in the fruit, they begin differentiation early in the ontogeny of the organ, and new ones are formed during its maturation.

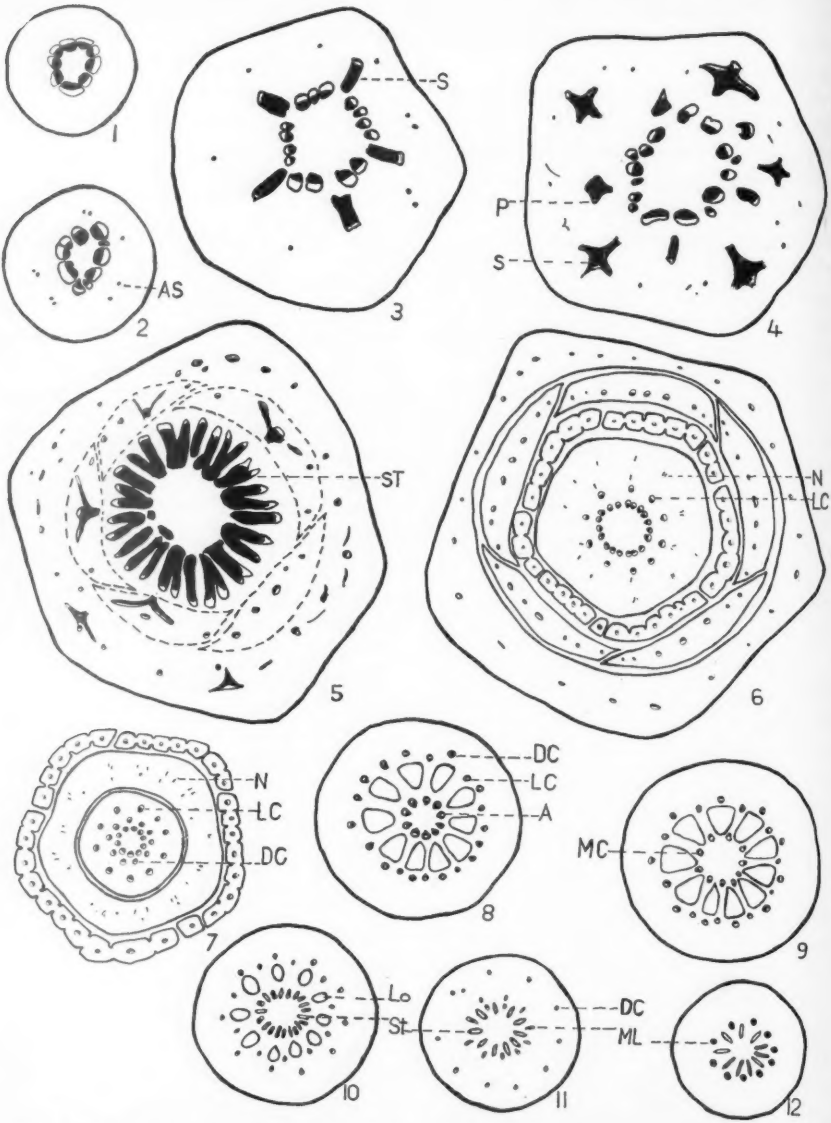
An endodermis is not well differentiated, but the transition to small cells of the pericycle usually is abrupt. The pericycle in pedicels of flowers is entirely composed of thin-walled cells, but in older stages small groups of strongly lignified cells occur adjacent to the phloem.

Some five to nine collateral bundles make up the vascular cylinder (fig. 1). Cambial activity is largely fascicular but finally may become interfascicular as well. Secondary thickening is not great, and most of the elements produced are not lignified, even when the fruit is half mature.

The pith usually does not exceed the width of the cortex. Its cells are isodiametric, and intercellular spaces are small. Many cells of the pith are lignified when the fruit is mature.

RECEPTACLE

The transition from pedicel to receptacle is gradual. The latter is consider-



FIGS. 1–12.—Figs. 1–4, cross-sections of pedicel and receptacle showing arrangement of perianth traces. *as*, accessory sepal traces; *s*, sepal trace; *p*, petal trace. Fig. 5, section showing divergence of stamen traces (*st*). Figs. 6, 7, sections at level of base of ovary showing order of divergence of nectary bundles and two lowest carpel traces. *lc*, lateral carpel (septal) traces; *dc*, dorsal carpel traces. Fig. 8, section of ovary below divergence of marginal carpel bundles. *a*, axial or receptacular bundle. Fig. 9, section of ovary above end of residual vascular tissue showing inverted marginal carpel bundles (*mc*). Fig. 10, section above placenta showing position of stylar canals (*st*) and tops of locules (*lo*). Fig. 11, section through base of style showing convergence of marginal and lateral carpel bundles (*ml*) and position of dorsal carpel bundles (*dc*). Fig. 12, section through lower part of stigma.

ably larger in diameter than the pedicel, because the cells of the cortex and pith are larger and more numerous. There are two series of sepal traces. Below the bases of the sepals are several groups of small vascular traces. Some of these are not attached below, but others diverge from the vascular cylinder about 3 mm. below the bases of the sepals. All these bundles, accessory sepal traces (fig. 2), extend into the lateral part of the sepals. Main sepal traces diverge from the vascular cylinder at a level of bases of the sepals. In flowers with five sepals there are five main sepal traces (fig. 3), each of which has three branches. The median one of these branches is connected with the median bundles of a sepal, while the other two are curved upward and are joined with the lateral bundles of the adjacent petals.

Receptacular bundles are curved in such a way that the gaps above the main sepal traces are closed, and a whorl of petal traces diverges from the vascular cylinder at this level. These traces are equal in number to the sepal traces and alternate with them. Each petal trace has three branches (figs. 4, 5). The median one is connected with the petal bundles, while the two lateral ones extend into the marginal portions of two adjacent sepals. There is considerable anastomosis and branching of the lateral branches of the sepal and petal traces beneath the bases of sepals and petals.

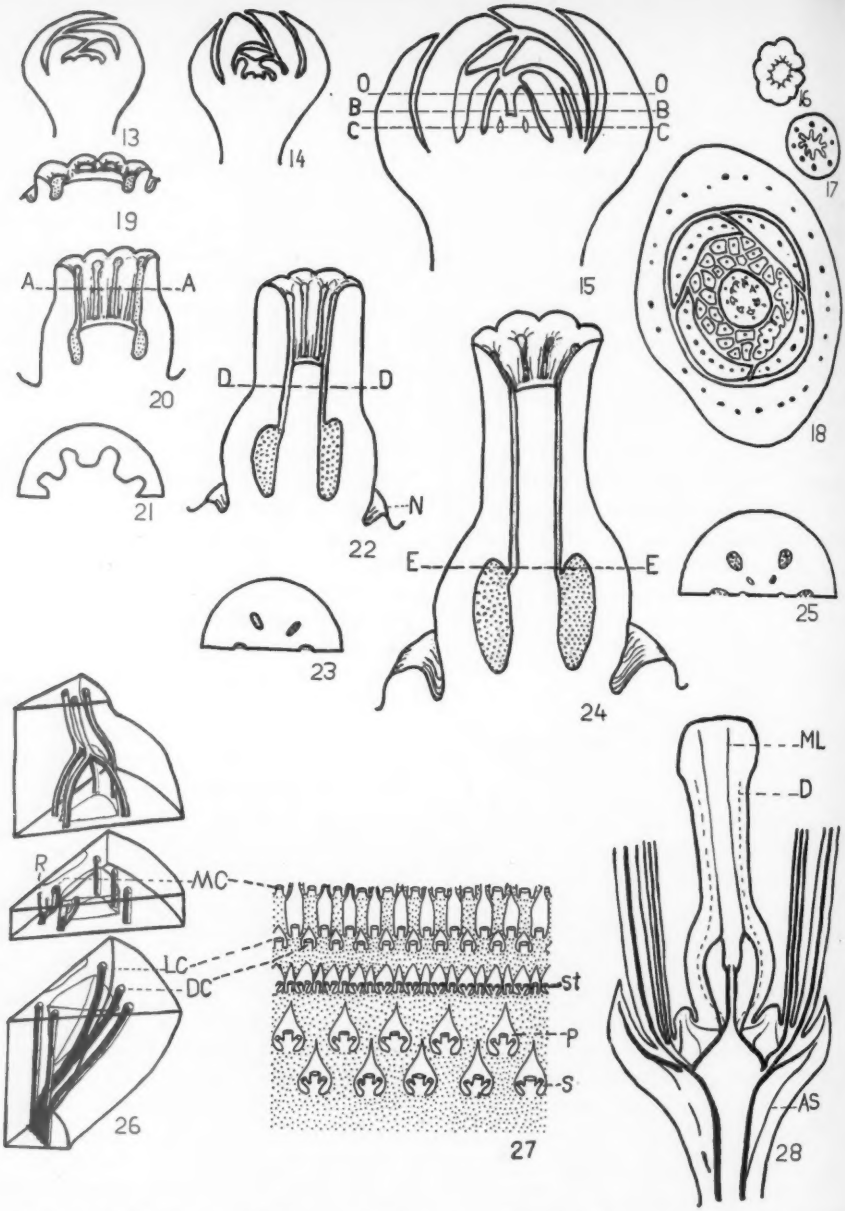
A whorl of fifteen to twenty stamen traces (fig. 5) diverges from the bundles of the axis above the level of the gaps of the petals. Approximately ten of these traces are forked immediately, so that there are about twenty-five to thirty bundles which extend into an equal number of stamens. In flowers which have five sepals usually the traces of the episepalous stamen are unbranched, but they appear at the same level as other stamen traces, not below them, as PENZIG (38) reported for orange.

Theoretically each carpel has one dorsal bundle, two lateral or septal bundles (which are usually undiverged from those of adjacent carpels), and two similarly undiverged marginal bundles. The lateral carpel bundles diverge from the vascular cylinder just above the level of the gaps of the stamens (fig. 6). There is no specific location of these bundles in alternation with those of other floral parts below, because of the variable number of stamens and carpels.

The dorsal carpel bundles diverge from the vascular cylinder just above, and alternate with, the gaps above the lateral bundles (fig. 7). But the gaps above the dorsal carpel bundles extend several millimeters, to the level of the center of the ovary (figs. 26, 27). At this level the marginal carpel bundles diverge into positions alternate with the dorsal ones, directly inward from the septa (figs. 9, 26). The receptacular bundles terminate at the level of the divergence of the marginal carpel bundles or extend slightly above and close some of the gaps above the dorsal bundles (fig. 27).

CALYX

The primordia of the three to six sepals are first to appear and originate separately in a whorl, although almost immediately meristematic activity begins between the primordia, and growth of the whole zone produces the synsepalous portion of the calyx which arches over the apex of the receptacle until the developing petals force it aside (figs. 13, 14). The calyx usually is less than 1 cm. long, but its base becomes thick and broadened slightly into the saucer-shaped structure which persists when the fruit is mature.



FIGS. 13–28.—Figs. 13, 14, longisections of flower bud showing origin of stamen and carpel primordia, respectively. Fig. 15, longisection of older flower bud prior to formation of gynoecial disk. Figs. 16, 17, cross-sections of carpels at *OO* and *BB*, respectively. Fig. 18, cross-section of flower bud at *CC*. Figs. 19–25, diagrams of successive stages in development of carpels showing ontogeny of stylar canals and nectary. Fig. 21, cross-section at *AA;* fig. 23, at *DD;* fig. 25, at *EE*. Fig. 26, diagram of sector of young fruit showing arrangement of main vascular bundles of one locule. *r*, receptacular residual tissue; *mc*, *lc*, *dc*, marginal, lateral, and dorsal carpel bundles. Fig. 27, diagram representing vascular axis of flower slit lengthwise and laid out flat; position of various traces somewhat idealized. *st*, stamen traces; *p*, petal trace; *s*, sepal trace. Fig. 28, longisection of flower diagrammatically showing relation of vascular bundles. *ml*, extension of both marginal and lateral carpel bundles; *d*, dorsal carpel bundle; *as*, accessory sepal trace.

The vascular bundles of the calyx are extensions of three whorls of traces. In flowers which have five sepals the median portion of each contains branches of a main sepal trace, while bundles in the lateral portions of each are connected with branches of adjacent petal traces and with accessory sepal traces. The five to eight bundles present at the base of each sepal are branched and anastomosed sparsely along their courses through the body of the sepal. Cells of the mesophyll are compact near the apexes of the sepals but near their bases are separated by large intercellular spaces. A few oil glands occur on the abaxial sides of the sepals.

Corolla

The petals originate as a whorl of crescent-shaped primordia. They alternate with the sepals and develop individually (figs. 15, 18). Their epidermal cells early become papillose, especially near the apices and edges. These papillae result in overlapping and interlocking of the petals, so that the corolla forms a closed chamber. Opening of the corolla is accompanied by shrinkage and plasmolysis of these papillose cells. In petals of a mature flower the cells of the mesophyll are isodiametric, and small intercellular spaces are abundant.

The five to eight bundles present in the base of a petal are branched repeatedly and anastomosed near the margins. These bundles are branches of two whorls of traces in the receptacle. Bundles of the median portion of each petal are connected directly with a median branch of a petal trace, while the lateral bundles of a petal are connected with lateral branches of adjacent sepal traces. While this is doubtless the fundamental pattern of vascular supply of the petals, and is in agreement with the findings of others (**38, 56, 61**), great variation exists. In corollas with three or four petals several branches from two adjacent petal traces may be present in one petal. Even in corollas with five petals, which is assumed to be the ancestral state, the bundles of the lateral portion of a petal may be connected with a complex of branches of sepal and petal traces, so that the true attachment is not discernible.

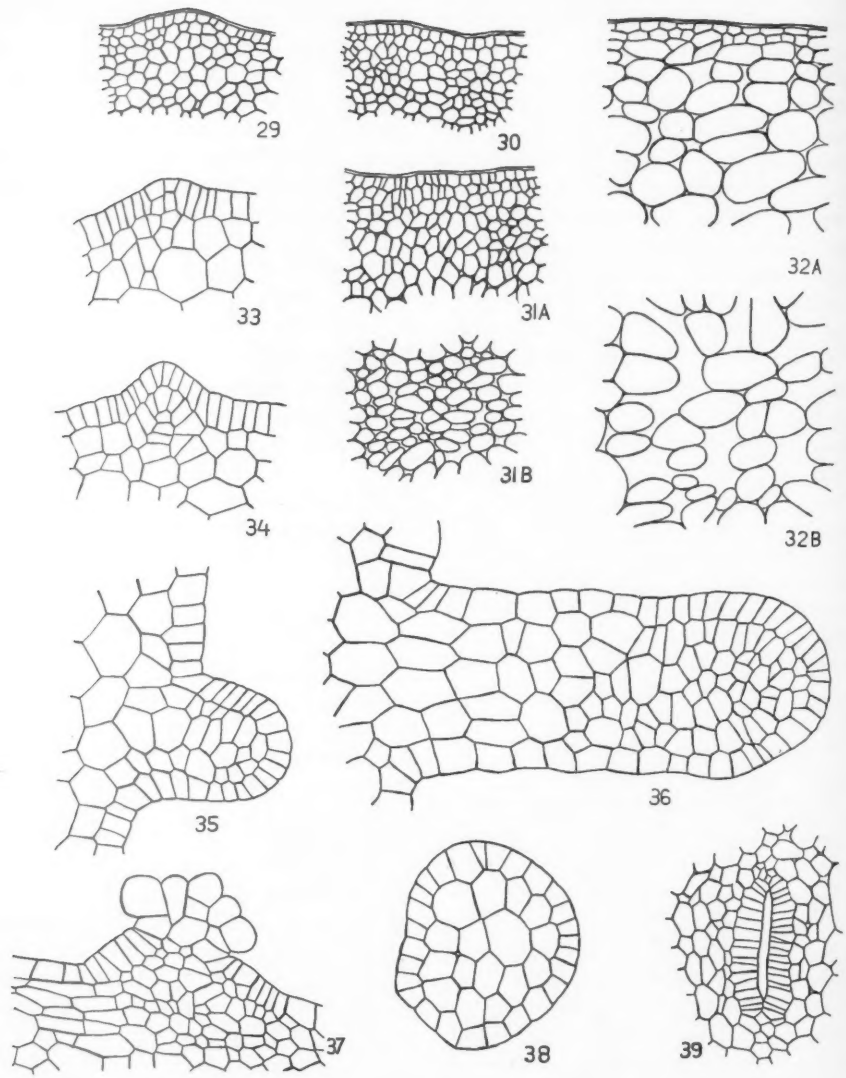
Stamens

The stamens originate after the petals have grown sufficiently to arch over the apex of the axis. Stamen primordia are dome-shaped and in one whorl. Although numerous young stages were observed, there was no evidence that the primordia of episepalous stamens originate before the other ones, in the manner reported for *C. aurantium* (**37, 38**). While some of the twenty-five or more stamens are separate almost to their bases, others are joined laterally in groups of three to seven, and only the upper 3–4 mm. of the filaments is separated (**15, 28, 48, 49**). Some filaments are noticeably longer than others, but they are not correlated with definite positions.

Each stamen has a single vascular bundle which extends into the anther. In the Eureka lemon the stamen traces all diverge from the vascular axis in a zone of 12 μ, and this can hardly be conceived to be two nodes. While this observation is in accord with other data (**37, 56, 61**), Penzig (**38**) considered the stamens of three other species of *Citrus* to arise in two whorls discernible by two whorls of vascular traces.

Pistil

Early ontogeny.—The carpels originate as a whorl of about nine crescent-shaped primordia located laterally near the apex of the axis. Cell multiplication



FIGS. 29–39.—Figs. 29–32*B*, section of ovary wall and pericarp in stages from prebloom to mature fruits, with diameters of 0.65, 4, 13, and 45 mm. Figs. 31*B* and 32*B* taken from inner pericarp adjacent to dorsal carpel bundles. Figs. 33, 34, primordia of juice sacs from radial longitudinal section of ovary of unopened flowers. Fig. 35, young juice sac as seen in longisection of ovary at time petals abscise. Fig. 36, median section lengthwise of juice sac from young fruit 10 mm. in diameter. Fig. 37, median longisection of one of carpellary emergences which failed to develop into juice sac; from fruit 24 mm. in diameter. Fig. 38, cross-section of juice sac at same age as fig. 36. Fig. 39, cross-section through stylar canal of mature flower. Cf. older stage, fig. 43.

occurs between bases of the primordia simultaneously with that in the primordia and the axis in such a way that the carpels develop with their margins undiverged from the margins of adjacent carpels or the axis. In early stages, therefore, the carpels are chambers open at the top (figs. 19, 20). At first the abaxial portions of the carpels grow faster than the adaxial portions and the axis, resulting in the formation of a depression in the apex of the developing pistil (fig. 22). The terminal portions of carpels develop into the stigma, while the style is developed from the apex of the receptacle, together with the upper portions of the adjacent carpel tissue. During elongation of the carpels the tops of the chambers become constricted into stylar canals (figs. 22–25). General meristematic activity of cells of the lower portions of the carpels and adjacent axis enlarges the locules. Early in their ontogeny ovule primordia develop on both margins of the carpels, and as the locule is increased in length new primordia originate below and above those formed earlier. At the time the flower opens the pistil consists of a receptacular core undiverged from the whorl of carpels. The placentation is axile, and a stylar canal extends from each locule to the stigma.

VASCULAR BUNDLES.—The dorsal carpel bundles differentiate early, and they are represented by one strong bundle for each carpel. They occur in the ovary wall at positions opposite the locules and in the outer part of the style opposite the stylar canals. The dorsal carpel bundles terminate just below the stigma (fig. 28). The marginal (ventral) carpel bundles differentiate at about the same time. They diverge from the receptacular bundles at the level of the center of the ovary and may equal the number of locules (fig. 9). Marginal carpel bundles are located in the axis of the ovary at positions opposite the septa, then are curved outward near the tops of the locules, where each anastomoses with a lateral carpel bundle (figs. 10, 11) and forms one concentric bundle which extends to the stigma (figs. 11, 12, 28). Lateral carpel bundles differentiate after the dorsal bundles. They are opposite the septa, curve inward, and are anastomosed with marginal bundles near the tops of the locules. In many instances the lateral bundles are in pairs along parts of their courses, but elsewhere the two bundles of the adjacent carpels are undiverged. The dorsal and lateral carpel bundles are much branched. These lesser vascular bundles extend outward at various angles from a complex network. Free ends of the latter bundles occur in the outer part of the ovary wall, and none of them extends into the style. At the time the flower opens the various bundles of the ovary are not well differentiated, and their histology is discussed in connection with the fruit.

OVARY.—When the flower opens the ovary is about 3 mm. in diameter and 5 mm. in length. The ovary wall consists of the median portions of the lower parts of the carpels and is about thirty cells thick. The outer cells of the ovary contain plastids which develop chlorophyll soon after the flower opens. Parenchyma of the middle portion of the ovary wall contains intercellular spaces but elsewhere is compact.

Oil glands are numerous in the outer part of the ovary wall. They are first detected as groups of cells whose cytoplasm is very dense and whose walls have begun to separate. Further development of an oil gland involves disintegration of a group of cells and formation of a cavity containing oil and other products. Large glands may extend halfway across the

ovary wall, as in other species of *Citrus* (8, 20, 58, 59). First initiation of glands takes place early in the development of the carpels and not after the flower is open, as reported for a Russian variety of Mandarin (20). Oil glands also occur in all other parts of the flower except the disk and the stamens, as others have noted (60). They are not associated with the adaxial surface of carpels, although BONAVIA (9) considered the juice sacs to be such.

The septa are composed of compact parenchyma and epidermis of adjacent carpels.

The lower part of the axis of the ovary consists of compact parenchyma and the receptacular bundles and placental traces. In the upper part the parenchyma is less compact, and in addition to the marginal carpel bundles stylar canals are present, the epidermal cells of which have dense protoplasts (fig. 39).

The anatropous ovules are turned in various directions. Some of their bundles diverge from marginal carpel ones, while the lower bundles usually diverge from the receptacular ones below the points where marginal carpel bundles diverge. The stylar canals are continuous with the locules above the bases of the uppermost ovules, and there are hairlike outgrowths of the epidermal cells at this point (fig. 41), each consisting of one slender cell or a filament of a few cells.

STYLE.—The style is composed of small unelongated parenchyma among which occur the two alternating whorls of vascular bundles, a few peripheral oil glands, and the stylar canals (figs. 11, 12). All pollen tubes observed were located in stylar canals.

STIGMA.—The stigma is globose and usually has an irregular central cleft. Cells at the surface are deeply staining and elongated at right angles. Near the edge of the stigma these cells comprise only one layer, but over the apex the stigmatic surface is composed of parallel filaments of two or three cells. In some stigmas oil glands occur just underneath the surface, near the ends of vascular bundles.

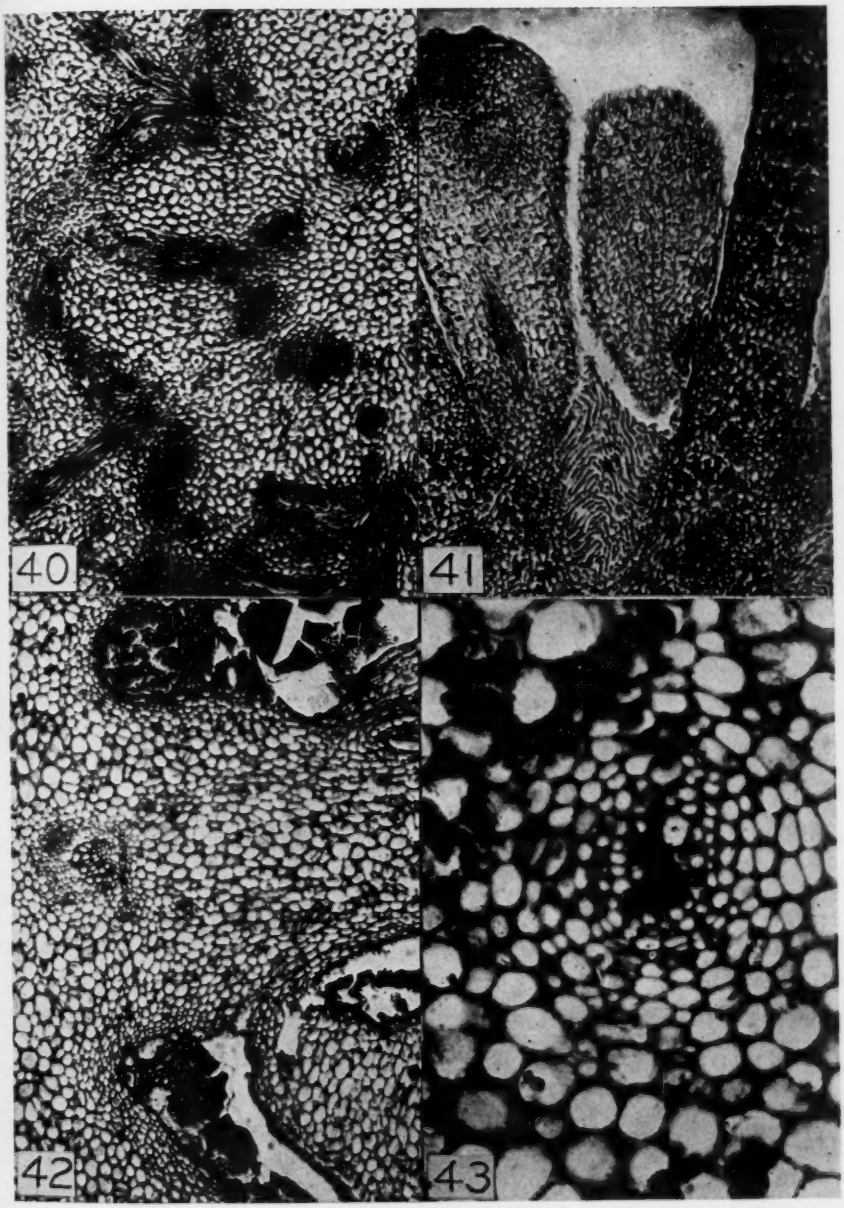
GYNOECIAL DISK

About the time the carpels begin to constrict at their tops, a ring of tissue above the bases of the stamens and below the carpels becomes meristematic and develops a massive, somewhat cup-shaped structure, the gynoecial disk, which completely encircles the base of the ovary (fig. 28). It is compact, stains densely, and its cells are exceedingly small and thin walled. Guard cells are present in its epidermis. Numerous small vascular strands are present (figs. 6, 7) and, contrary to reports (56) for other species of *Citrus*, usually are not attached directly to the vascular cylinder or to stamen traces, but to lateral carpel traces. This nectary does not develop further.

FRUIT

After the petals and sepals abscise, meristematic activity continues in various parts of the ovary. The top of the style disintegrates, and the fruit then develops from the ovary and base of the style (42).

RIND.—Cell multiplication in the ovary wall proceeds rather uniformly until the fruit is 10 mm. in diameter and the pericarp 3 mm. thick. In such young fruits the cells of the pericarp are thin walled, and intercellular spaces are minute and limited to the middle portion, where cells on the whole are twice the size of those of the outer and inner few layers. In subsequent stages the pericarp differentiates into three zones: an outer one of compact cells containing the



FIGS. 40–43.—Cross-sections. Fig. 40, of receptacle of flower bud showing one petal trace emerging from axis at position alternate with sepal traces which are branched into three strands each. Fig. 41, of ovary of open flower showing placental hairs at junction of stylar canal. Fig. 42, two undiverged inverted marginal carpel bundles. Mucilage occurs among placental hairs. Fig. 43, of stylar canal from apex of young fruit showing mucilaginous content of space. Cf. younger stage, fig. 39.

oil glands, a middle zone of spongy tissue, and an inner pericarp which is only a few cells thick and is attached to the juice sacs. Aside from the vascular bundles and epidermis, the whole pericarp is parenchymatous. The rind consists of the outer two zones of the pericarp, and although it does not separate easily from the pulp of the fruit, as does that of certain other species of *Citrus*, it is histologically distinct.

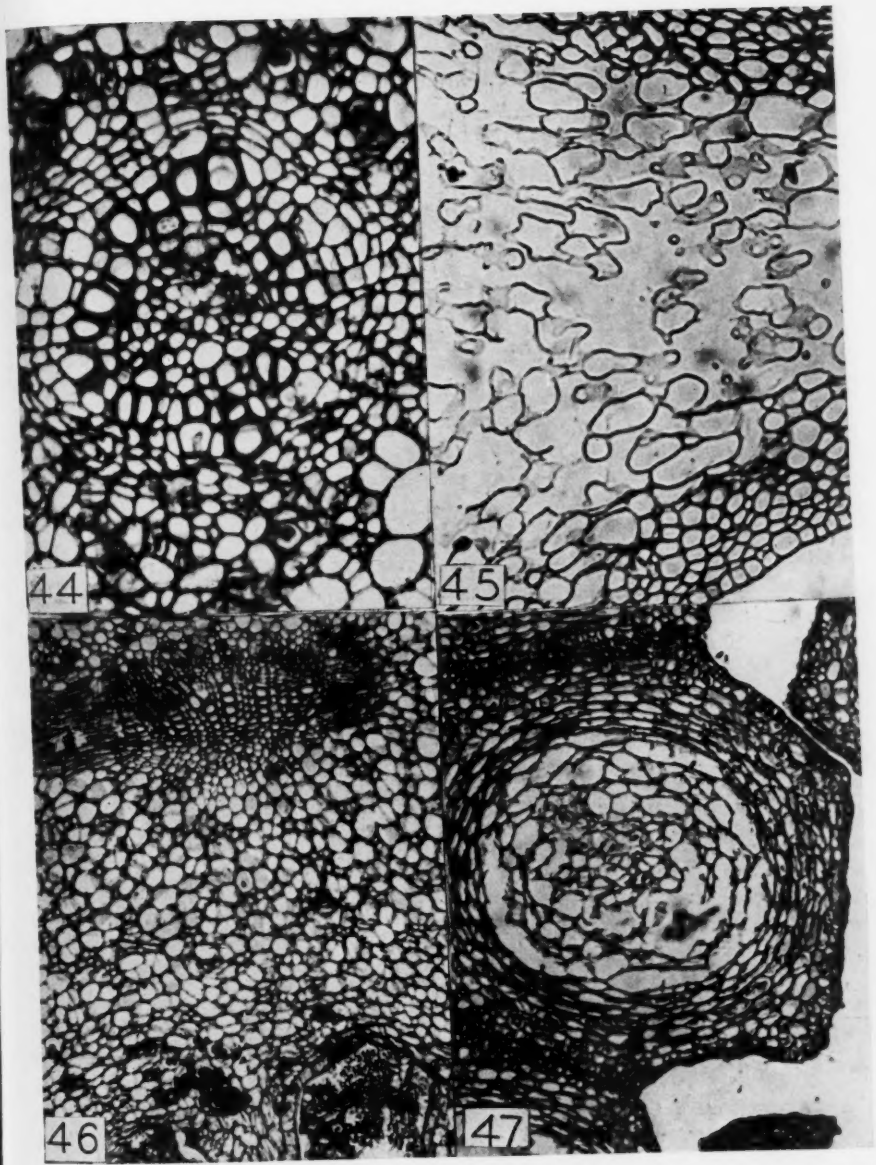
Stomata are present but are not abundant in the epidermis. Cells just underneath the epidermis are small and compact (figs. 29–31), but there is progressive increase in their size and of intercellular spaces farther inward (fig. 31*B*). These cells are isodiametric until mature, when they are slightly elongated parallel to the epidermis (fig. 32*A*). Oil glands are abundant underneath the epidermis, but they usually are separated from one another by eight or more parenchymatous cells, and many vascular bundles end near these glands. While some of the glands are formed during the early stages of development of the carpels, many new ones continue to develop, until the fruit is 15–20 mm. in diameter.

The spongy layer is notably developed (fig. 32*B*) when the fruit is 25 mm. in diameter and occupies about one-third of the width of the pericarp. Parenchymatous cells of this area are very thin walled and before the fruit is mature assume irregular shapes resembling leaf mesophyll. Intercellular spaces in mature fruits appear to equal or exceed the volumes of the cells. The demarcation between this zone and the outer one is gradual, but change to the small compact cells of the inner pericarp is abrupt.

All the vascular bundles of the ovary wall occur in the rind. During development there is increase in number as well as in size and differentiation of all the bundles. The dorsal and lateral carpel bundles change most. In young fruits these bundles are collateral and a cambium is present (fig. 46). But after the fruit is about 12 mm. in diameter the parenchymatous cells adjacent to the xylem portions of the vascular bundles become progressively meristematic toward the protoxylem of each bundle, and from their derivatives additional phloem, xylem, and cambial elements are differentiated (fig. 44).

INNER PERICARP AND JUICE SACS.—The inner pericarp is developed from the outer adaxial epidermis of the carpels, together with a narrow band of subepidermal cells. It is merged with the outer parts of the septa. The epidermal cells are similar to those of the outer pericarp, except that cutin is very thin and stomata are absent. The parenchyma adjoining the epidermis is compact and there are no oil glands. This zone contains no vascular bundles, although dorsal and lateral carpel bundles occur just outside it.

Development of the inner portion of the pericarp includes formation of the club-shaped emergences, the juice sacs, which extend into the locule. Primordia of these structures appear just before the flower opens, as reported for *C. aurantium* (**41, 5**). Their initiation after flowering has also been reported (**18, 31**). At first a primordium consists of a group of about five epidermal cells which have elongated radially and formed a small mound protruding into the locule. Primordia were observed only along the outer boundary of the locules and not on the septa. PENZIG (**38**) found the same condition in orange, but in *C. hystrix* he observed juice sacs attached to outer parts of the septa. In early stages of growth anticlinal divisions of epidermal cells of the primordium and division of



FIGS. 44–47.—Cross-sections. Fig. 44, of marginal carpel bundle from fruit 13 mm. in diameter. Cambium has become a cylinder and secondary xylem and phloem are present on all sides of primary xylem. Fig. 45, of inner portion of septum of mature fruit. Fig. 46, of portion of inner pericarp of 13 mm. fruit, showing position of dorsal carpel bundle and bases of juice sacs. Fig. 47, of juice sacs from fruit 24 mm. in diameter.

subepidermal cells take place. Early periclinal divisions of the epidermis were observed, but the emergence does not originate wholly from the epidermis (figs. 33, 34). During elongation of juice sacs there are repeated periclinal divisions of the epidermal cells at the tip, so that most of its cells are of epidermal origin. Young juice sacs are cylindrical and about five cells in diameter (figs. 35–38). They grow across the locules and project among the developing ovules when the fruits are 10 mm. in diameter. Each developing sac, at some point along its length, increases in diameter two or more times by cell multiplication in that area (fig. 47). This produces the familiar club shape of the emergence. There is little cell multiplication after the fruit is 20 mm. in diameter, however, and later enlargement of juice sacs results from increase in cell size. In mature fruits parenchymatous cells at the center of the juice sacs have very thin walls with minute intercellular spaces. Protoplasts have large vacuoles, and, by the use of Sudan 4, oil substances were detected in the protoplasts, as reported by DAVIS (14), as well as in the spaces formed when some cells break down. The epidermal cells which bound the mature juice sac are small, and many of them have thickened cell walls when the fruit matures.

Not all primordia develop into juice sacs, but some enlarge only slightly and their terminal cells become enlarged and globose, and cell divisions cease (fig. 37). TSCHIRCH (58) thought these papillose cells were the source of the mucilage so abundant in the locule of the young fruit of *C. vulgaris*, but Eureka lemon shows no evidence that mucilage is secreted from abortive emergences any more than from the developing juice sacs. Mucilage was not found in the locules of older fruits.

SEPTA.—Cells of the septa are markedly uniform, and cell multiplication continues until the fruit is some 20 mm. or more in diameter, when intercellular spaces become conspicuous in the median portions of the septa. This band of spongy parenchyma is merged on either side with the compact subepidermal cells and the epidermis of the carpels. In mature fruits, cells of the spongy area are stretched radially into long ellipsoidal shapes and are distinctly individual (fig. 45).

CORE.—The core develops from the axial portion of the ovary. Cell divisions increase the number to about 150 cells across the axis at the level of the placenta. In fruits that have attained 15 mm. in diameter small intercellular spaces begin to appear among the parenchymatous cells, and in subsequent development the central area of the core becomes spongy, especially above the level of the divergence of marginal bundles, where the spaces between vascular bundles is greatest. Similarly there is great development of intercellular spaces between the vascular bundles and the locules. This spongy area is continuous with that of the septa (fig. 45).

All the conducting hairs at the juncture of the stylar canals and locules become coated with mucilage and begin to decompose by the time the fruit is about 20 mm. in diameter (fig. 42). The core includes the lower parts of the stylar canals. Cells lining the canal itself usually do not break down with maturation of the fruit, but their cells become less compact. Their protoplasts become much less dense and resemble those of adjacent parenchyma (fig. 43). The canal itself contains mucilaginous material.

Vascular bundles of the lower part of the core increase in size. Fascicular cambium produces several rows of sec-

ondary tissues, and interfascicular cambium is produced between some of the bundles. Lignified tissues include a few annular elements, but for the most part they are composed of spiral tracheids 0.1–0.5 mm. in length. The thin-walled elements are elongated cells that stain densely, but no sieve plates were found. Above the ends of the axial bundles the marginal carpel bundles occur in the outer part of the core. In their early development they are endarch collateral, but during maturation they are changed by the same kind of cambial activity described previously in the dorsal and lateral bundles. The development of this condition occurs first toward the top of the locules. The ovule traces, which at blossoming time are minute, develop into conspicuous concentric bundles when the fruit is mature.

Discussion

Flower

While the flower of *Citrus* conforms with none of Shaffner's (47) floral types, it is not a complex one; yet in the literature several features have led to confusion in the application of morphological criteria.

The fact that the perianth is supplied by three series of vascular bundles rather than by two from the vascular axis raises a question in regard to the accessory sepal traces. If they are considered vestiges of other flowers of the inflorescence, in agreement with the ideas of Arber (2), then the concept of the flower as a pseudanthium is involved. Although this interpretation is conceivable, it seems unnecessary in the case of the lemon. Accessory sepal traces might be considered merely secondary bundles arising in connection with the enlarged receptacle. The fact that their provascular elements are present as early in ontogeny as those of the receptacular bundles would not favor this idea. A third possibility is that these bundles represent lateral or marginal sepal traces. This concept is supported by the facts that they occupy normal positions for such bundles and that a few of them were found attached to bundles that diverged as main sepal traces. It may be unusual for lateral traces to diverge from the vascular cylinder below the main dorsal ones, but that condition has been described by Penzig (38) in leaf traces of certain oranges and seems also to be true of the lateral abaxial bundles of the carpels of Eureka lemon.

It has been assumed by Tillson and Bamford (56) that the stamens in *Citrus* belong to more than one whorl. Payer (37) and Penzig (38) thought there were two whorls in *C. aurantium*. But in Eureka lemon it has not been possible to observe either the three ages of stamen primordia or two whorls of stamen traces which the latter investigators used as evidence. The coherence of stamens in indefinite groups does not shed light on the issue, and the criterion of alternation cannot be used in this regard in lemon because of the number of carpels. In complete agreement with van Tieghem (61), observations on the anatomy of the lemon indicate there exists one whorl of stamens, although in a flower with thirty stamens they conceivably may represent two whorls of five single stamens and two whorls of five forked stamens, all "compressed" into one level. To apply Wilson's (63) idea of primitiveness of forked stamens in the interpretation of the stamens of Eureka lemon would add to the confusion.

Opinions concerning the cup-shaped nectaries of *Citrus* have followed two widely different lines. Partly because they found its vascular bundles attached to stamen traces, Tillson and Bamford

(56) considered the so-called gynoecial disk to represent a vestigial stamen whorl. This view seems not to be substantiated by the facts that in Eureka lemon many, if not most, of the bundles present in this structure are connected with those of the main receptacle or with the lowermost carpel traces, and also that it originates after the carpels have attained considerable size. If the gynoecial disk is a vestigial structure, there is no more evidence for considering it as derived from stamens than from another whorl of carpels. More in accord with anatomical facts is the view that the disk arises merely as a proliferation of the axis, owing to continued meristematic activity accompanying growth of the gynoecium. This might account for the indefinite attachment of the vascular bundles supplying this structure and for its origin out of acropetal order. Views similar to this were held by BAILLON (5), PAYER (37), PENZIG (38), and others.

In the foregoing description the tacit assumptions have been that the flower represents the terminal portion of one shoot and that the carpels are foliar in nature, in the manner expressed by KOZO-POLJANSKI (30), PARKIN (36), and NEWMAN (34). Nothing has been observed in the developmental anatomy of lemon to make it desirable to apply the concept of sterile and fertile carpels of SAUNDERS (45) nor to resort to the so-called "new morphology" of THOMAS (52) or THOMPSON (53, 54, 55), who deny the existence of the carpel as such. Other contradictory interpretations regarding the angiosperm carpel are those of TROLL (57) and GREGOIRE (24), and it is not pertinent here to discuss them, for that has been done by others (4, 6, 21, 33, 64, 30, 3). GREGOIRE, however, has erred in one anatomical point, for in the fruit of Eureka lemon differentiation of the carpel is not different from that of a foliage leaf, either in regard to the vascular bundles or to other tissues.

How much of the gynoecium is composed of axis tissue is of interest in species of *Citrus* because various workers, notably SAUNDERS (46) and OSAWA (35), have alluded to the prolongation of the axis in the Navel orange, and others (7, 17) have referred to this condition in other families. On evidence of ontogeny and the positions of the bundles in the style, the axis might be conceived to extend well up the style and to occupy there most of the space surrounded by the stylar canals. But there is no way to distinguish accurately how much of this tissue is carpel margins, and there is little residual vascular tissue in the Eureka lemon, and none of it extends into the style.

The epidermis of the stylar canals leads from the stigma to the placental surface of the locules. It is therefore strange, as ARBER (3) and TILLSON and BAMFORD (56) point out, that JOSHI (27) considered these structures in angiosperms generally to be vestigial vascular bundles. Because of the stylar canals, each carpel has the superficial form of a cupule whose terminal portion is constricted, as suggested by THOMAS (52) for carpels of certain simpler pistils; but this fact does not indicate homology with such structures present in the Caytoniales.

Placental areas offer no obstacle to a foliar interpretation of the carpels. The origin of younger ovules, both above and below the older ones, is explained by the general meristematic activity during development of the ovary as a whole. The continuity of the vascular bundles of the lowest ovules with the receptacular bundles is directly related to adnation of carpel margins to the receptacular axis

and does not require their interpretation as axial ovules, as BUGNON (10) did for those in *Begonia*. The hairs at the juncture of the stylar canal are similar in nature to the papillose epidermal areas of the petals and the stigma.

The coherence of vascular bundles of adjacent carpels is pronounced, both as regards marginal and lateral (septal) carpel bundles, but the dorsal carpel bundles diverge from the vascular cylinder above the lateral carpel bundles. This might be expected in *Citrus*, since the traces of a foliage leaf—according to PENZIG (38)—diverge in a similar manner; that is, lateral leaf traces diverge from the vascular cylinder below the level where the main dorsal leaf traces are separate from the vascular cylinder.

The presence of concentric bundles in the style need not be confusing when it is recalled that each is an extension of two bundles; and one of these, the marginal carpel bundle, has its xylem and phloem in inverted positions because of the position of the margins of the carpels.

FRUIT

To consider the rind of lemon a torus, as DE CANDOLLE (15) did for certain species of *Citrus*, contradicts all concepts of the angiospermous carpel except those of SAUNDERS (45), because most of the carpel bundles occur in the rind. The same may be said of BONAVIA'S (9) concept of the rind as a whorl of sterile carpels. It is improbable that either worker conceived carpels to be polymorphic, but it seems much more likely that their ideas were based upon the ease with which the rind separates from the pulp, and that they ignored the vascular anatomy of the fruit.

When the structure of the rind is viewed in comparison with that of a foliage leaf, it becomes evident that the line where rind can be separated from the inner pericarp coincides with the spongy mesophyll tissue present in many foliage leaves. The fact that this zone of intercellular space development in the carpels of lemon is near the upper rather than the lower epidermis can be explained on ecological bases. The separation of the sectors of the pulp from one another and from the core likewise is made possible by the copious development of intercellular spaces adjacent to the inner epidermis of the carpels. The small amount of cell disintegration observed along these lines of cleavage seemed coincidental with cell separation rather than with other causes involving secretion of specific enzymes.

It is perhaps unwise to homologize juice sacs with epidermal hairs, because this may obscure the fact that juice sacs are derived from more than the epidermis of the carpel, a fact well established in at least three species of *Citrus* by POULSEN (41), PENZIG (38), BIERMAN (8), and by the present observations. It is also improbable that the juice sacs are derived from oil glands (9). Presence of oil in cells of the juice sacs is similar to other cells of the pericarp, and the occurrence of spaces in older juice sacs is probably due to tension resulting from osmotic forces. There are no structures of Eureka lemon with which the juice sacs may be homologous.

Summary

1. In Eureka lemon the succession of the whorls of the flower parts is acropetal. The nectary or gynoecial disk originates after the carpels.

2. The courses of the vascular bundles and the general histological features of the floral parts are described.

3. The gynoecium is considered a whorl of carpels around the apex of the

receptacle which extends past the level of the placenta.

4. Development of the fruit involves a period of cell multiplication throughout the ovary, followed by one of cell differentiation. Differentiation in the pericarp produces three zones: (*a*) an outer exocarp which is compact and contains oil glands, the flavido; (*b*) the mesocarp which is spongy and becomes most highly developed close to the inner pericarp, the albido; (*c*) the inner pericarp which is compact and from which the juice sacs diverge.

5. Primordia of juice sacs appear just before opening of the flower and occur on the adaxial surface of each carpel. After the juice sacs grow across the locules, a portion of each enlarges greatly in diameter. Cells in the center of these areas contain oil and are extremely delicate. Spaces containing gelatinized material in the juice sacs are considered incidental to high turgor and not to action comparable to gland formation.

6. Separation of the sectors of the pulp from one another and from the rind and core is made possible by cell separation and the formation of a spongy area in the tissue adjacent to the adaxial surfaces of the carpels.

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INFLUENCE OF SULPHUR DEFICIENCY ON METABOLISM OF BLACK MUSTARD

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 549

SCOTT V. EATON

(WITH ONE FIGURE)

Introduction

The writer has for some time been studying the effects of sulphur deficiency on the metabolism of plants. The project as originally outlined involved three plants. Since leguminous and cruciferous species are especially high in sulphur, the former on account of the high protein content and the latter owing to various sulphur compounds in addition to proteins, it was planned to study a representative of each family. And for comparison a plant from a third family was to be investigated. The three plants chosen after considerable preliminary work were the Manchu soybean, a representative of the Leguminosae; the black mustard, belonging to the Cruciferae; and the Mammoth Russian sunflower, a member of the Compositae.

The results of the effects of sulphur deficiency on two of the plants have already been published, the soybean (2) and the sunflower (3). Other phases of the subject which have been investigated are the effects of sulphur deficiency on the volatile sulphur content of the black mustard (5) and the relation of the sulphur content of the seed and seed weight to the effects of sulphur deficiency on the growth of the sunflower (4). The present paper reports the results for black mustard, *Brassica nigra* (L.) Koch.

Since this paper completes this entire project, the discussion section summarizes and compares the effects of sulphur deficiency on the growth and metabolism of plants, with special reference to the three plants already mentioned.

Methods

GROWING THE PLANTS.—In choosing a cruciferous plant for this study, kale, rape, and white mustard were grown with plus-sulphur and minus-sulphur nutrient solutions in connection with the work on the soybean (2). The weights of the tops and roots were obtained and the top-root ratios calculated, but no analyses were made. The effects of sulphur deficiency on black mustard and white mustard were compared in the summer of 1941 as part of the volatile sulphur experiment (5). Much greater reduction in growth was obtained in the case of the former species, and it was decided to use this plant.

The methods of growing the plants were essentially the same as previously described (3). The main modification made was that a more dilute nutrient solution was used. In growing the plants during the spring and summer for the volatile sulphur study (5), some of them developed certain chlorotic effects and malformations of the leaves which did not seem normal. So in the autumn of 1941 an experiment was tried which involved varying the concentration of the nutrient solution and also the frequency

of its application. The growth period was October 4 to November 14. The results are given in table 1.

Although, perhaps due to seasonal differences, the plants of none of the solutions—even the most concentrated—developed the abnormal characteristics just mentioned, the plants of the half-strength nutrient solution added daily are considerably larger than those of any other series. Since the plants were grown during the short days of autumn, however, it was thought best in the present work to use a somewhat stronger concentration, and a solution three-fourths of full strength was used. Table 2 gives its composition. Boric acid and manganese chloride at a concentration of 0.25 p.p.m. of each of the elements, boron and manganese, were kept constantly in the nutrient solution, which was added daily. A solution of ferric citrate at a concentration of 1.0 p.p.m. of iron was sprinkled on to the sand twice a week, and zinc chloride and cupric chloride, at concentrations of 0.05 and 0.02 p.p.m., respectively, of zinc and copper, were applied every 2 weeks.

TABLE 1

GREEN WEIGHT (GRAMS PER 25 PLANTS)

SOLUTION	TOPS	ROOTS	ENTIRE PLANT
Full strength* added daily	55.5	10.3	65.8
Full strength added three times per week	58.0	11.3	69.5
One-half strength added daily	70.5	12.3	82.8
One-half strength added three times per week	57.0	8.3	65.5
One-fourth strength added daily	58.1	10.2	68.3
One-fourth strength added three times per week	42.5	6.8	49.3

* Full strength refers to a nutrient solution having an osmotic pressure of one atmosphere. The solution used was IR_3S_4 of the triangle (7). Because of the greater ionization, the diluted solutions would, of course, have an osmotic pressure greater in proportion to the full-strength solution than that indicated by the dilution.

TABLE 2

PARTIAL VOLUME MOLECULAR CONCENTRATION OF SALTS OF NUTRIENT SOLUTION

SOLUTION	KH_2PO_4	$Ca(NO_3)_2$	$MgSO_4$	$MgCl_2$
Minus-S	0.0034	0.0068	.....	0.0034
Plus-S	0.0034	0.0068	0.0034	.....

The seeds were planted on April 8, 1942, and the plants harvested May 11.

CHEMICAL METHODS.—At the time of harvest some of the plants of each set had formed flower buds. None of these were included in the sampling. Two hundred and thirty minus-sulphur and 101 plus-sulphur plants were taken for analysis. Only the stems were analyzed, since previous work (2) indicated that sulphur deficiency affects the stems of plants more than other parts. The stems were cut up finely, and duplicate 100-gm. samples were weighed. The tissue was extracted with 70 per cent alcohol, as in the previous work (3). This seems to be an efficient solvent. In the case of the nitrogenous compounds, most of the nitrogen is found in the extract; there is little in the residue (table 4).

The methods used in the determination of the nitrogenous fractions were essentially as previously described (2, 3). The determinations were made promptly to avoid changes such as those described by WEBSTER (17) and STUART (15). PHILLIPS' (10, 11) methods were used in the determination of amides and nitrates, with a few modifications. Five per cent H_2SO_4 instead of 10 per cent as suggested by PHILLIPS was used in the hydrolysis of the amides, and the ammonia was aspirated off with 0.65 per cent HaOH, as given by DAVIDSON and SHIVE (1). Ten per cent H_2SO_4 seems a rather high concentration for amide hydrolysis. In the determination of nitrates, the boiling

down to a small volume after the transference of the extract to the Kjeldahl flask was not done because it was thought that ammonia might be liberated from some of the nitrogenous compounds, owing to the increased concentration of the alkali.

Volatile sulphur was determined by the method of PLATENIUS (12) and the other sulphur fractions as previously described (3).

Results

The effects of sulphur deficiency on the size of the plants developed rather quickly. In less than 2 weeks after planting, the minus-sulphur plants were shorter and the leaves smaller than the plus-sulphur. Chlorosis of the leaves developed late. It was not until about 1 week before harvest that this could be detected; then the young upper leaves showed the symptom to a marked degree. Up to this time the leaves of the minus-sulphur plants were even greener than the plus-sulphur. Anthocyanin development in leaves and stems, hairiness of these organs, the pim-

TABLE 3

GREEN WEIGHT (GRAMS PER 100 PLANTS), TOP-ROOT RATIO

SOLUTION	LEAVES	STEMS	ROOTS	ENTIRE PLANT	TOP-ROOT RATIO	PERCENTAGE MOISTURE OF STEMS	PERCENTAGE DRY WEIGHT OF STEMS
Minus-S	342.6	129.1	47.9	519.6	9.847	92.726	7.274
Plus-S	660.2	507.6	82.5	1250.3	14.152	95.051	4.949

TABLE 4

NITROGEN FRACTIONS (IN PERCENTAGES)

SOLUTION	DRY MATTER	GREEN MATTER	DRY MATTER	GREEN MATTER	DRY MATTER	GREEN MATTER	DRY MATTER	GREEN MATTER
	TOTAL N		TOTAL ORGANIC N		70% ALCOHOL-SOLUBLE N		70% ALCOHOL-INSOLUBLE N	
Minus-S	6.112	0.445	2.906	0.218	4.520	0.329	1.592	0.116
Plus-S	6.143	0.304	2.285	0.113	4.391	0.217	1.752	0.087
	AMMONIA N		AMINO N		AMIDE N		NITRATE N	
Minus-S	0.237	0.0172	0.452	0.034	0.219	0.016	3.116	0.227
Plus-S	0.077	0.004	0.114	0.006	0.106	0.005	3.858	0.191

The only modification made in the carbohydrate methods was in the permanganate titration of cuprous oxide. This was made at room temperature, with the indicator ortho-phenanthroline, rather than at a temperature of 80° C. as recommended by SULLIVAN (16). The indicator fades so rapidly at the high temperature that it is rather difficult to detect the end point. This does not occur at room temperature, and yet the permanganate reacts readily.

ply character of the younger leaves, and the hardness of the stems and the stiff texture of the leaves were also prominent effects of sulphur deficiency. Figure 1 shows the difference in the size of the plants at the time of harvest. The photograph does not do justice to the difference in color.

In a number of cases the tips and edges of the young leaves of the minus-sulphur plants bent downward. This was not noticed in the case of the previous plants (2, 3) and is not considered a characteristic symptom, although McMURTREY (8) states that sulphur deficiency frequently causes the tips of the leaves of the tobacco to crimp downward, and STOREY and LEACH (14) describe the uprolling of the leaves of the tea bush as characteristic of the yellows disease of this plant. Their work shows rather definitely that this disease is due to a deficiency of sulphur.

FIG. 1.—Black mustard plants, May 10, 1942; 32 days from planting of seed.

The indications were that sulphur deficiency caused earlier reproduction. It has been mentioned that at the time of harvest a number of the minus-sulphur and plus-sulphur plants had formed flower buds. A count showed that this had occurred in a higher percentage of the former than of the latter. The phenomenon had been noticed in preliminary work on both the black and white mustard. The matter should be studied more fully, using a greater number of plants. As stated, none of the plants that had formed flower buds were taken for analysis.

It is evident from table 3 that sulphur deficiency very much reduces growth of the mustard plant. The stems are affected much more than the leaves and the roots. The top-root ratio of the minus-sulphur plants is relatively low, showing that the weight of the tops is reduced more than that of the roots, but of the two organs of the tops, the ratio is due much more to the stems than to the leaves.

Tables 3–9 give the data for the chemical composition of the plant. Because of the big difference in the amount of dry matter in the two sets of plants, the percentages are figured on both dry-weight and green-weight bases. The data are also calculated on the absolute basis.

NITROGEN FRACTIONS

Calculated as percentage of green matter, the minus-sulphur stems contain more than four times as much ammonia, five times as much amino acids, and three times as much amides as the plus-sulphur stems (table 4). All the other nitrogen fractions are higher in the sulphur-deficient stems, although the nitrates accumulate only to a slight extent. When the data are calculated on the dry-weight basis the differences are less and are reversed in the case of total nitrogen, 70 per cent alcohol insoluble nitrogen, and nitrate nitrogen. The ammonia was determined by vacuum distillation and therefore includes any other volatile bases present.

Table 5 gives the data of the nitrogen fractions calculated on the absolute basis. The much larger plus-sulphur stems

contain more of all the nitrogen fractions except ammonia and amino acids. The fact that the much smaller minus-suphur stems contain more of these compounds emphasizes their accumulation as a result of sulphur deficiency.

Carbohydrate fractions

On the green-weight basis the minus-sulphur stems contain only about one-fifth as much reducing sugars as the plus-sulphur, but they contain almost four times as much sucrose, six times as much starch, and also somewhat more acid-hydrolyzable carbohydrates (table 6). On the dry-weight basis the difference in reducing sugars is intensified but reduced in the case of the other compounds.

When the data are calculated on the absolute basis (table 7), the large plus-sulphur stems contain more of all the carbohydrates except starch. The percentage of this carbohydrate is so much higher in the small minus-sulphur stems that they contain more of it than the much larger plus-sulphur stems.

Table 8 records the percentage data for volatile sulphur, for the sulphur left in the extract, and for the residue after removal of the volatile sulphur by steam distillation. The determinations do not represent duplicates. It was not possible to attend to more than two pieces of the volatile-sulphur apparatus at the same time, so one determination was made on each of the two sets of plants on each of two consecutive days. The plants were of the same crop but were necessarily different plants. One of the plus-sulphur determinations was lost.

On the dry-weight basis the minus-sulphur stems contain from less than one fifth to less than one-tenth as much volatile sulphur as the plus-sulphur stems. The differences on a fresh-weight basis are less. The two percentages for

TABLE 5

NITROGEN FRACTIONS (GRAMS PER 100 PLANTS)

Solution	Total N	Total organic N	70% alcohol-soluble N	70% alcohol insoluble N	Ammonia N	Amino N	Amide N	Nitrate N
Minus-S	0.574	0.281	0.425	0.149	0.022	0.042	0.021	0.293
Plus-S	1.543	0.574	1.103	0.440	0.019	0.029	0.027	0.969

TABLE 6

CARBOHYDRATE FRACTIONS (IN PERCENTAGES)

Solution	Dry matter	Green matter	Dry matter	Green matter	Dry matter	Green matter	Dry matter	Green matter
	Reducing sugars		Sucrose		Starch		Acid-hydrolyzable carbohydrates	
Minus-S	0.318	0.023	0.549	0.040	2.404	0.175	10.495	0.764
Plus-S	2.303	0.114	0.230	0.011	0.608	0.030	9.657	0.478

the sulphur-deficient stems do not agree very well, owing mainly to the very small amount of sulphur present. The determinations varied by only 2.5 mg. of barium sulphate or 0.34 mg. of sulphur. The sulphur in the extract, in the residue, and total sulphur are also much lower in the minus-sulphur than the plus-sulphur stems. No sulphur at all was found in the residue of the former and very little in the latter. On the absolute basis (table 9) the large plus-sulphur stems contain much more of each of the fractions. Considerable additional data on the effects of sulphur deficiency on the volatile sulphur content of the black mustard are given in a previous paper (**5**).

TABLE 7

CARBOHYDRATE FRACTIONS (GRAMS PER 100 PLANTS)

SOLUTION	REDUCING SUGARS	SUCROSE	STARCH	ACID-HYDROLYZABLE CARBOHYDRATES
Minus-S....	0.030	0.052	0.226	0.986
Plus-S.....	0.579	0.058	0.153	2.426

TABLE 8

SULPHUR FRACTIONS (IN PERCENTAGES)

SOLUTION	DRY MATTER	GREEN MATTER	DRY MATTER	GREEN MATTER	DRY MATTER	GREEN MATTER	DRY MATTER	GREEN MATTER
	VOLATILE S		S IN EXTRACT		S IN RESIDUE		TOTAL S	
Minus-S.....	0.0045	0.0003	0.2171	0.0165	0.0000	0.0000	0.2216	0.0168
Minus-S.....	0.0088	0.0008	0.1229	0.0107	0.0000	0.0000	0.1317	0.0115
Plus-S.....	0.0480	0.0028	0.6450	0.0378	0.0355	0.0021	0.7286	0.0427

TABLE 9

SULPHUR FRACTIONS (GRAMS PER 100 PLANTS)

SOLUTION	VOLATILE S	S IN EXTRACT	S IN RESIDUE	TOTAL S
Minus-S....	0.0007	0.0335	0.0000	0.0342
Minus-S....	0.0013	0.0189	0.0000	0.0202
Plus-S.....	0.0199	0.2673	0.0147	0.3020

Discussion

In the previous papers (**2, 3**), rather detailed discussions have been given of the effects of sulphur deficiency on the appearance and chemical composition of the plant, the apparent explanation and interrelation of these effects, and their relation to the effects of the deficiencies of other elements. Since the present work on the mustard largely confirms the previous studies, these discussions will not be repeated here. Instead, a general review follows (*a*) summarizing the influence of sulphur deficiency on plants in general, (*b*) describing in what respects the effects differ in various plants, and (*c*) discussing the relation of sulphur deficiency to the volatile sulphur content of the mustard.

SUMMARY OF EFFECTS OF SULPHUR DEFICIENCY ON PLANTS

The main symptoms of sulphur deficiency are the yellow-green color of the leaves, the smaller leaves, and the thinner stems as compared with plants grown

with complete nutrient. The stems are usually hard and woody, and they usually have a low water content. Minus-sulphur plants generally exhibit great power of stem elongation. Flower and fruit development are likely to be early.

Sulphur deficiency also results in the accumulation in the plant of the soluble organic nitrogenous compounds such as amino acids and amides; the inorganic nitrogenous compounds, ammonia and nitrates; and the polysaccharides, especially starch. The plant is also high in the soluble organic sulphur compounds, although these have not been studied very completely in this series of studies. The sugars, especially the reducing sugars, are usually low as compared with plus-sulphur plants.

Because of the great accumulation in minus-sulphur plants of the soluble organic sulphur and nitrogenous compounds, an abnormal amount of the latter being amides, because of the reduction in sugars of these plants, and because of their great power of stem elongation, these data have been interpreted as indicating proteolysis and the reutilization of the resulting sulphur and nitrogenous compounds in stem elongation. Sulphur deficiency has been found to reduce reductase activity (**6**). In this way the accumulation of nitrates may be accounted for, and the resulting decrease in protein synthesis may account for the accumulation of starch. Poor nitrate utilization would seem to account for the reduced chlorophyll development, and the hardness of the stems is probably to be explained by their high content of starch and to a less extent of acid-hydrolyzable carbohydrates.

If this is the correct explanation, it is a rather unique mechanism that enables the plant to carry on the processes of proteolysis and starch accumulation at the same time. Proteolysis is usually accompanied by a decrease in all forms of carbohydrates; slow nitrate reduction, by their accumulation. In a sulphur-deficient plant reducing sugars are usually low but starch is high. A minus-sulphur plant is in many respects similar to a minus-nitrogen plant, but the latter accumulates all kinds of carbohydrates because of poor protein synthesis resulting from the deficiency of nitrates. There is little proteolysis; most of the nitrogen is in the form of protein rather than of soluble compounds. There is limited power of stem elongation.

In the previous studies (**2**, **3**), reference has been made to another method of explaining, in part at least, the accumulation in a sulphur-deficient plant of soluble organic nitrogenous compounds. All plant proteins apparently contain sulphur. The species specificity of proteins is a well-established concept. Calculations comparing the organic nitrogen of seed and plant show that a minus-sulphur plant synthesizes considerable organic nitrogen. But owing to the great deficiency of the sulphur-containing amino acids, condensation of the nonsulphur-containing amino acids and the amides to the proteins characteristic of the species is largely prevented; hence they accumulate.

But certain features can hardly be accounted for by reduced protein synthesis of a sulphur-deficient plant. Among these are the accumulation of soluble organic sulphur compounds, the great reduction in reducing sugars, and the power of elongation. It would seem that proteolysis is going on in these plants more rapidly than in the plus-sulphur ones, and that this accounts—in part at least—for the much larger amounts of the soluble organic nitrogenous and sulphur compounds. The rather large proportion of amides to amino acids in the nitrogenous material would also seem to be in line

with this conclusion, although this may perhaps be accounted for in other ways. It is likely, therefore, that both proteolysis and reduced protein synthesis are factors in the accumulation in the sulphur-deficient plant of soluble organic sulphur and nitrogenous compounds.

Differences in effects of sulphur deficiency on plants

While plants are rather uniform in the nature of sulphur-deficiency symptoms, they differ in the speed with which these symptoms develop. The sunflower (3) early showed both size and color differences. The difference in size in the mustard was soon evident, but chlorosis developed late. The effects of sulphur deficiency on both size and color developed late in the soybean (2). Nightingale (9) found that the symptoms developed gradually in the tomato.

Such differences are no doubt to be expected. The sulphur requirements of plants vary. Also the amount of sulphur introduced as an impurity in the various experiments was no doubt different. Another paper (4) gives the main sources of sulphur as an impurity. These are the sulphur in the seed or plant used, the sulphur in the air, and the sulphur in the salts of the nutrient solution. For plants grown in the open the sulphur in rainwater constitutes another source.

Plants vary in both the nature of the chlorosis and its gradation. Usually the chlorosis is uniform over the entire leaf, but in the case of the tea bush (14) the leaves become mottled. As to the gradation, the upper leaves generally show the chlorosis first. This is true of soybean (2), mustard, tobacco (8), and apparently of the tea bush (14). No gradation was evident in the sunflower (3), and Nightingale (9) found that the lower leaves of sulphur-deficient tomato plants become yellow-green first.

Increased cell-wall thickness is usually a prominent effect of sulphur deficiency. Nightingale (9) emphasizes it in the tomato. The stems were not examined anatomically in this series of studies, but it was noticed that the minus-sulphur soybean (2) and mustard stems were harder than the plus-sulphur ones. This did not seem true of the sunflower (3). Often the stiff texture of minus-sulphur leaves is prominent. This was true of the mustard, and Storey and Leach (14) describe it as a prominent symptom in the tea bush. This was not evident in the soybean and the sunflower.

Stem elongation is generally a prominent feature of sulphur-deficient plants, but the degree of prominence varies. Nightingale (9) found that the minus-sulphur tomato plants were as tall as or taller than the plus-sulphur. The sulphur-deficient soybean (2) and sunflower (3) plants elongated remarkably but were decidedly shorter on the average than the plants grown with complete nutrient. Stem elongation was not marked in the mustard until shortly before flower-bud formation. Since the plant naturally elongates at this time, it is impossible to separate the effects of sulphur deficiency and the reproductive processes.

The usual situation in regard to the chemical composition of sulphur-deficient plants has been given in a previous paragraph. The main variation from this is that, while generally the reducing sugars are low in these plants as compared with those grown with complete nutrient, these sugars accumulate in the stems of the tomato (9) together with the other carbohydrates. The relative amount of sucrose also varies in different plants. Minus-sulphur tomato and mustard stems are high in sucrose as compared with plus-sulphur stems; on the other hand, minus-sulphur sunflower (3) and

soybean (2) stems are relatively low in this sugar.

It is rather hard to account for these differences in the carbohydrate content of sulphur-deficient plants. It may be due to a variation in the relative influence of proteolysis and lessened reductase activity, both of which are phenomena of plants grown with a deficiency of sulphur. Proteolysis is usually preceded or accompanied by a reduction in the amounts of the carbohydrates present; interference with reductase activity results in the accumulation of carbohydrates owing to poor protein synthesis. On this basis the influence of the lessened reductase activity would seem to be predominant in the tomato, causing the accumulation of all forms of carbohydrates. But in the other plants studied, proteolysis is accompanied by the usual reduction in carbohydrates, at least to the extent that the reducing sugars are low, interference with reductase activity operating to cause the accumulation of starch and usually also to a certain extent of the acid-hydrolyzable carbohydrates. In regard to sucrose, sometimes the latter influence operates and it accumulates; sometimes the former, and its content decreases.

Influence of sulphur deficiency on volatile sulphur content

Of the three plants studied in this investigation, the mustard is the only one containing an appreciable amount of volatile sulphur. Tests indicated that the sunflower (3) and the soybean (2) contained little if any. But since in a previous paper (5) considerable data have been given as to the amount of volatile sulphur in the organs of the black mustard and the effects of sulphur deficiency and other conditions on the amount present, the subject will be discussed only briefly here.

The volatile sulphur compound of the seed, and presumably of the plant, is allyl mustard oil, $CH_2CHCH_2 \cdot NCS$. The leaves contain more of the compound than the stems and the young leaves more than the older leaves. Growing the plant in a nutrient solution lacking sulphur results in the almost complete lack of development of volatile sulphur. The leaves taste flat; they do not have the sharp, acrid taste characteristic of mustard, and analyses show that very little volatile sulphur is present. The present work confirms this as regards the stems (table 8). No work was done on the leaves.

Both nitrogen and sulphur are components of allyl mustard oil. It might perhaps be expected that growing the plants in solutions lacking these elements would result in decreased pungency. But while minus-sulphur leaves contain very little volatile sulphur (5), and the volatile sulphur content of plant tissue varies directly with the sulphur content of the growth medium (13), minus-nitrogen leaves are even more pungent than plus-nitrogen ones (5). The nature of the regulatory mechanism causing the difference is not known. However, with this difference in the relationship of sulphur deficiency and nitrogen deficiency to pungency, it is hardly surprising that minus-nitrogen leaves contain more volatile sulphur than plus-nitrogen ones. The sulphur content of the nutrient solution in which the two sets of plants grew was the same. The former leaves were much smaller than the latter. They had available, therefore, much more sulphur per unit of tissue.

Summary

1. The effects of sulphur deficiency on the size of black mustard plants was evident early, but chlorosis of the leaves appeared late. The main symptoms were the short plants, the yellow-green color of the upper leaves, the small leaves and the

thin stems. The stems were hard and the leaves had a stiff texture. Anthocyanin development in leaves and stems, the hairy nature of these organs, and the pimply character of the young leaves were prominent. The weight of the tops was reduced more than that of the roots, causing a low top-root ratio; but of the tops the stems were affected more than the leaves. Stem elongation was not prominent.

2. The sulphur-deficient stems were low in moisture. They were very high in ammonia, amino acids, and amides. Nitrates accumulated to a certain extent. The minus-sulphur stems were very low in reducing sugars, but they were much higher in sucrose and starch and also somewhat higher in acid-hydrolyzable carbohydrates than the stems of the plus-sulphur plants. Little volatile sulphur was found in the sulphur-deficient stems.

3. The chemical composition of the sulphur-deficient stems seemed to be due mainly to the combined influences of proteolysis and poor reductase activity. The breakdown of proteins accounted mainly for the accumulation of ammonia, amides, and amino acids, although this was no doubt partly the result of reduced protein synthesis owing to deficiency of sulphur-containing amino acids. Slow reduction of nitrates caused their accumulation and the resulting poor nitrate utilization for the accumulation of sucrose, starch, and acid-hydrolyzable carbohydrates. The accumulation of these carbohydrates accounted for the hard stems and stiff texture of the leaves. Proteolysis is usually accompanied by decrease in the carbohydrates. This was true in the present work to the extent that the reducing sugars were low in the minus-sulphur stems.

4. The effects of sulphur deficiency on plants in general are summarized and compared.

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NUTRITIONAL EFFECTS OF BORON ON GROWTH AND DEVELOPMENT OF THE SUNFLOWER

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 550

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Introduction

Although a number of possible roles of boron in the nutrition of plants have been suggested, sufficient data are not available to lend more than tentative support to any of them. SKOLNIK (15) and REHM (11) indicate that boron serves to regulate the accumulation of ions from the nutrient substrate. WARINGTON'S (20) results suggest that boron is especially correlated with the intake of calcium. That such intake is lessened in the absence of boron has been corroborated with the soybean (9). SHIVE (14) found, in maize and in the broad bean, that—in the absence of boron—the total calcium in the plant was not detectably reduced, but that the soluble calcium was much less.

SMIRNOV (16) concluded that boron acts to regulate the normal growth of the various parts of the tobacco plant, chiefly the stem. The influence of boron on the growth of roots and leaves is less marked than on the other parts of this plant. The effect of boron deficiency on the vascular system suggests a relationship between boron and translocation of the nutrient materials. MORRIS (10) uses this to explain the fact that citrus plants grown under boron deficiency showed proportions of organic and inorganic materials in the leaves and fruits different from the proportions in plants to which boron was supplied.

To date, the influence of boron on the accumulation of materials from the nutrient solution has been studied to a considerable extent by means of analyses of the nutrient medium from which the roots of the plants absorb the materials. A direct analysis of the plant itself has many advantages, however, especially if it can be divided into parts before analysis, so that local accumulation can be ascertained. It was with this in mind that the present work was undertaken.

Methods

The methods used in growing the plants were essentially those employed by EATON (3). On May 4, 1941, sunflower seedlings (*Helianthus annus* L., variety Mammoth Russian) 3 days old were transplanted to quartz sand in glazed crocks of about 1250-cc. capacity. SHIVE'S (13) three-salt solution was used, except that ferric citrate instead of ferric tartrate was employed. Only c.p. and analytical-grade chemicals were used. When the plants were well established, they were thinned to one per crock, leaving in all fifty plants.

On May 24, when the plants were 23 days old, differential treatment was started. The plants were divided into fourteen groups of three plants each and the others discarded. The groups were paired in such a way that the members of each were as nearly similar to the members of the adjacent group as possible.

The sand of all crocks was rinsed thoroughly with distilled water before the

plus- and minus-boron treatment was begun. Half the plants received nutrient solution containing added boron at a concentration of 0.75 p.p.m.; the other half had only such boron as may have occurred as impurities.

The plants were harvested 6 days after starting the treatment. They were divided into (1) stem tips, (2) leaves and petioles, (3) stems, and (4) roots. First the stem tips of a minus-boron sample were removed, selecting the approximate level where blackening of the stem tissue appeared. The tips of the stems of the corresponding plus-boron plants were then harvested, the cut being made at the point on the stem closely corresponding to the place where the minus-boron stems were cut. Since the plus-boron stems had elongated much more than the others, it was not possible to estimate this point accurately. In every case the cut was made in the fifth internode above the cotyledons.

The leaves and petioles were harvested and all above-sand portions of the plant weighed. Most of the sand was removed from the roots by rinsing with salt water, followed by thorough rinsing—first with tap and then with distilled water. All the parts were dried to constant weight at 90° C. and reweighed. The sand left on the roots was largely removed after drying by blowing the dry material. The samples were ashed in platinum crucibles in a muffle furnace at 600° C. The sand left on the roots after this treatment was determined as the hydrochloric acid-insoluble portion of the ash.

Calcium was determined in an aliquot of the ash by means of the Official Methods (1). MINARIK and SHIVE (9) have pointed out that precipitation of the silicon dioxide is not necessary, and accordingly this step was omitted.

The method of statistical analysis as described by SNEDECOR (17) was used in determining the significance of the data. Although the plants were arranged in paired replications as described, most of the analyses were made as pooled data.

Results

The plants were entirely healthy in appearance during the entire growing period before treatment was started. Twenty-four hours after placing the plants on minus-boron treatment, they showed the first indications of injury. This consisted of a pubescent grey appearance of the terminal buds and newly expanding leaves. No stem elongation occurred in these plants from the time minus-boron treatment was started. On the day of harvesting, the younger leaves were curled downward, the base was lighter green than the tip, and the tissue had become brittle. Black longitudinal streaks were visible at the time the tip of the stem was cut. Microscopic examination of similar plants indicated that these were the vascular bundles which had failed to differentiate normally.

Table 1 summarizes the data. The fresh weights of all the above-ground parts of the plant, except the leaves, were heavier in the case of the plus-boron than in the case of the minus-boron plants. No significant difference in the fresh weight of the leaves of the two groups is indicated.

The plus-boron plants contained a higher percentage of moisture. The minus-boron plants tended to become flaccid in warm clear weather, apparently not absorbing sufficient moisture to keep up with transpiration. The morning of May 30, when harvesting was done, was cool and cloudy, however, and the minus-boron plants were turgid.

The percentage of ash in the minus-boron plants was lower in the stem tip

TABLE 1

EFFECTS OF BORON SUPPLY ON SUNFLOWER PLANT

Data	Plant part															
	Stem tip			Stem			Leaves			Root			Total			
	Treatment		Difference	Treatment		Difference	Treatment		Difference	Treatment		Difference	Treatment		Difference	
	+B	−B		+B	−B		+B	−B		+B	−B		+B	−B		
Fresh weight (gm.)	25.00	16.04	8.96±1.56	37.94	27.09	10.85±1.97	15.7	16.30	−0.6 ±1.0	.....	.....	.....	78.31*	59.79	18.52±2.86	
Moisture (%)	90.57	89.61	0.96±0.15	94.30	92.95	1.35±0.10	91.96	91.10	0.86±0.19	.....	.....	.....	92.67*	91.50	1.17±0.11	
Dry weight (gm.)	2.34	1.67	0.68±0.14	2.16	1.92	0.24±0.13	1.27	1.46	−0.19±0.10†	1.39	1.17	0.22±0.05	7.13	6.23	0.91±0.24	
Ash (%)	16.36	14.53	1.83±0.27	16.7	17.00	−0.3 ±0.4	21.47	21.66	−0.19±0.65	22.7	21.4	1.3 ± 0.9	18.78	18.28	0.50±0.16	
Total ash per plant (gm.)	0.38	0.25	0.14±0.04	0.35	0.32	0.03±0.03	0.27	0.32	−0.05±0.02†	0.30	0.25	0.05±0.02	1.31	1.16	0.15±0.04	
Total combustible material per plant (gm.)	1.96	1.45	0.51±0.17	1.74	1.59	0.14±0.15	1.01	1.25	−0.24±0.09†	1.03	0.91	0.12±0.05	5.66	5.26	0.40±0.20	
Calcium in ash (%)	11.73	11.47	0.26±0.22	5.28	4.68	0.60±0.18	13.30	14.00	−0.72±0.26†	9.32	6.60	2.7 ±0.43	9.81	9.22	0.59±0.18	
Total calcium per plant (mg.)	45.30	28.40	16.9 ±4.80	18.40	15.20	3.20±1.83	36.18	45.27	−9.1 ±2.83†	28.82	16.70	12.1 ±1.64	128.94	109.67	19.3 ±6.70	

* Value does not include root. † Data paired in analysis.

and total plant. The total ash in the roots, stem tip, and entire plant was lower in the case of the minus-boron plants but significantly higher in the leaves.

The percentage of calcium in the ash of the minus-boron stems and roots was significantly lower than in the plus-boron. The reverse was true for the leaves, the plus-boron ones containing a lower percentage of calcium than the minus-boron. The percentage of calcium in the entire plant showed a significant difference in favor of the plus-boron group. The stem tip and root of the minus-boron plants contained less calcium, while the leaves contained more.

The total combustible material was determined as the difference between the dry weight of the material and the weight of ash (table 1). The data show that the weight of material lost in ashing the stem tip and root was greater in the plus-boron plants. On the other hand, the weight of material lost in ashing the plus-boron leaves was significantly less.

Plants grown under conditions similar to those under which the plants used in analysis were grown, and which were then transferred to treatment with distilled water, showed injury to the terminal portions of the stems. These injuries could not be distinguished from minus-boron symptoms, except that they progressed much more slowly. A few days after the symptoms of the stem tip appeared, the lower leaves of these plants became yellow. These eventually withered, and the yellowing and gradual death progressed toward the higher leaves. This symptom was not displayed by the minus-boron plants. Treating the plants which had been on distilled water with a solution containing 0.5 p.p.m. of added boron did not appear to affect the lower leaves, but recovery of the stem tip took place. This recovery was similar to that of minus-boron plants when transferred to complete nutrient solution, except that it was not so rapid.

Discussion

While the plus-boron plants remained completely healthy in appearance during the entire growth period, the minus-boron plants developed distinct symptoms of injury upon their transfer to minus-boron conditions. These symptoms consisted of total cessation of growth of the stem tip and change to the characteristic grey color of the young leaves. The appearance of the discolored and misshapen leaves and stem tips of the plants suffering from boron deficiency is distinctive. SCOFIELD, WILCOX, and BLAIR (12) illustrate the upper portion of a sunflower plant showing characteristic minus-boron symptoms.

Considerable significance may be attached to the observation of the plants transferred to distilled water. SOMMER and SOROKIN (18) found that in general the absence of boron and of certain other elements caused symptoms similar to those induced by the absence of boron alone. The present results suggest that boron is the first of all nutrient elements to become deficient, and that to this extent it limits the utilization of all other nutrients. The rapid appearance of minus-boron symptoms in plants having the other essential elements now acquires further significance.

The percentage of water in the tissues of the two lots does not agree with the findings of MINARIK and SHIVE (9). They found that when soybean plants were grown from seed under different levels of boron, the plants higher in percentage of boron were lower in moisture. While at first the present results seem to conflict with their data, the probable explanation is that their plants were de-

veloped under an almost unchanging boron level throughout life. In the present case the plants were grown under an adequate boron supply and then transferred to a deficient medium.

The data at hand support three generalizations.

1. EFFECT OF BORON DEFICIENCY ON WEIGHT INCREASE.—The reduction in growth of root and stem tip is striking. It is in agreement with the findings of JOHNSTON and DORE (7) but does not substantiate SMIRNOV'S (16) assertion that the influence of boron on the growth of the tobacco root is not marked. The length of the stem was considerably greater in the plus-boron plants, for it had elongated during the time that the minus-boron plants showed no growth. This is reflected in the fresh weight of the plus- and minus-boron stems. Apparently this elongation was accomplished largely by means of vacuolization of the cells, without considerable increase in solids, for the dry weights do not differ significantly. The data indicate that the parameter of the dry weight of the minus-boron leaves is at least as great as, and probably greater than, that of the plus-boron leaves.

2. PERCENTAGE ASH AND COMBUSTIBLE MATERIAL.—The evidence indicates that the percentage of ash in the stem tip was lowered by the removal of boron, and that the percentage of combustible material was accordingly increased. No other differences in the ash of the various parts are shown, but the average for the entire plant shows a significant difference in favor of the plus-boron plants. It is possible that had the plants been grown in water culture, a difference in the percentage of ash of the roots could have been determined. The sand clinging to the roots constituted an interfering factor which was never completely eliminated. HILL and GRANT (6) state that in turnip the roots of high-boron plants are lower in ash than are those with boron deficiency. The total ash and combustible material is a correlative of the dry weight and the percentage of ash of the plant parts. The data indicate that even though the percentage of ash in the stem tips of the minus-boron plants is less than in the plus-boron plants, the total of material lost in ashing (as well as of ash) is less. This is caused by the fact that the total weight of the stem tips of the minus-boron plants is considerably less than of the plus-boron plants. In the roots also, the minus-boron plants are lower in ash and in combustible material, but the reverse seems to be the case in the leaves. It seems improbable that this is caused by the removal to the leaves of materials from the roots and stem tips, for most of the plant nutrients are fixed in a form not readily transportable. The only other explanation, assuming that the differences between the plus- and minus-boron leaves are not caused entirely by chance, is that after the plants were transferred to minus-boron solution the net accumulation of inorganic ions from the nutrient medium was hastened in the leaf and the loss of phytosynthates reduced.

3. AMOUNT OF CALCIUM IN THE ASH.—No differences in the percentage of calcium in the ash of the stem tips could be observed. The ash of the minus-boron leaves was higher in calcium than that of the plus-boron leaves, while in the stem, root, and total plant it was significantly lower. The fact that no differences in the percentage of calcium in the stem tips of the plants could be observed indicates that boron does not have a direct effect upon calcium utilization by the cells of this part of the plant. Other workers have not observed the difference in calcium in the total plant, but since it

is small it could be detected only by controlled replications. MARSH and SHIVE (8) and SHIVE (14) report that in maize and in the broad bean the availability of a boron supply is not correlated with the amount of calcium in the plant, but that there is a strong correlation between boron and soluble calcium.

Apparently boron is essential for the normal distribution of materials through the various parts of the plant. In the absence of boron, the materials ordinarily used in the growth of the root and stem tip accumulate in the leaf. Furthermore, as has been observed by others, boron is essential for the formation and maintenance of the vascular system. CHANDLER (2) concluded, after observation of boron deficiency in a number of species in the Cruciferae, that boron is intimately associated with differentiation of cells. HAAS and KLOTZ (5) have observed in citrus, ELTINGE (4) in maize, and WARINGTON (19) in the broad bean, that the vascular system is not normal in plants deprived of boron. In the present experiment the phloem of the minus-boron plants appeared to be filled with a gum-like substance, in both the younger and older portions of the stem. No deterioration of the xylem was observed in the mature stem, but the secondary xylem failed to differentiate, apparently remaining parenchymatous. In the young stem most of the xylem region was devoid of tracheae. These facts, together with the data for the weights of the different parts of the plant, strongly indicate that boron is directly necessary for the maintenance of the vascular system, and that, in the absence of boron, materials cannot pass freely from one part of the plant to another. The data here presented constitute direct supporting evidence to this theory, previously suggested by others.

SCOFIELD, WILCOX, and BLAIR (12) suggest the use of indicator plants for determining whether the crop production of a field will be increased by boron fertilization. They suggest that the amount of boron in the leaf of a sunflower plant grown under standard conditions be used as the index of boron deficiency, but the method is unwieldy and probably not entirely accurate. The data in the present experiment suggest that the interrelationship between boron availability and the weight of the various portions of the plant is of practical value in determining when boron fertilization is desirable. The average quotient leaves/remainder of plant was 0.220 for the plus- and 0.308 for the minus-boron plants. This relationship may not hold under field conditions, or be sufficiently constant for use as a measure of boron deficiency, but the data suggest that the possibility is worth consideration.

Summary

1. Sunflower plants showed typical boron-deficiency symptoms promptly upon transfer from complete nutrient solution to one deficient in boron.

2. Plants transferred from complete nutrient solution to distilled water developed symptoms typical of boron deficiency. Upon supplying these with a dilute solution of boric acid, 0.5 p.p.m. of boron, partial recovery occurred.

3. Boron was found necessary for growth of the stem tips and roots. Inconclusive evidence was obtained that the leaves of plants were heavier if the plants were deprived of boron during the later part of the growth period.

4. The stem tips of the minus-boron plants contained a smaller percentage of ash. Elsewhere in the plant, differences in percentage of ash could not be observed.

5. No difference in the percentage calcium in the ash of the stem tips of the plus- and minus-boron plants could be observed. The ash of the leaves of the minus-boron plants contained more calcium, and the ash of the stem, roots, and entire plant contained less.

6. Available evidence supports the hypothesis that boron, because of its importance in the maintenance of the vascular system, is instrumental in regulating the distribution of materials through the various parts of the plant. The calcium determinations are in accord with the view that there is a relationship between boron availability to the plant and the utilization of calcium.

The writer is indebted to DR. SCOTT V. EATON, whose assistance and suggestions have helped to make this work possible. Acknowledgment is also due DR. CHARLES A. SHULL for his many suggestions while the experiment was in progress.

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DIFFERENTIATION OF AVOCADO BLOSSOM BUDS IN FLORIDA

PHILIP C. REECE[1]

(WITH THIRTEEN FIGURES)

Many species of cultivated deciduous fruit trees have been studied to determine the time of floral initiation, but less is known regarding the time of fruit-bud differentiation in evergreen tropical and subtropical fruit trees. Such knowledge is important because of the possibility of influencing differentiation by cultural practices.

Most deciduous fruit trees (for example, apple, pear, plum, and cherry) initiate flowers during the summer following their period of vegetative growth in the spring. Most of the floral organs are formed before the leaves fall and emerge the next spring after a period of dormancy. Fruit trees of the tropics and subtropics, which usually exhibit several distinct cycles of vegetative growth within the season, are not in such a category. Frequently it is difficult to distinguish between a flower bud and a purely vegetative one before it opens, either from its size or position. ABBOT[2] has shown that blossom-bud differentiation takes place in citrus during initiation of growth in the spring or upon resumption of growth at any other season as the result of a period of favorable environmental conditions. Flowers may thus appear on axes which did not exist a few months earlier.

Terminal and lateral buds of the avocado were collected at monthly intervals, beginning October 8, 1940, from trees of the Lula and Nabal varieties, growing on the grounds of the United States Horticultural Field Laboratory at Orlando, Florida. Some variation in the stage of development existed over various parts of a tree, so that each collection was taken from branches which represented as nearly as possible the average stage of bud development at the time of collection. From these branches the terminal buds and the four to six lateral buds nearest the terminal portion were taken for sectioning. The buds were killed in 70 per cent alcohol, imbedded in paraffin, and sectioned longitudinally at 10–15 μ.

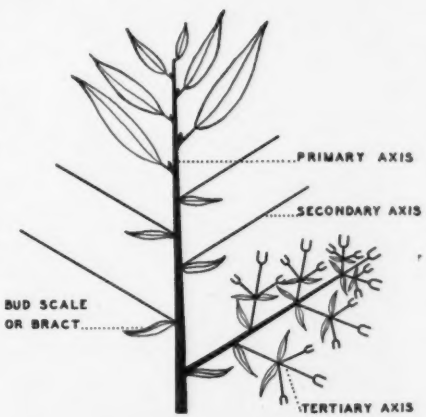

FIG. 1.—Diagram of floral and vegetative growth developed from terminal or lateral winter buds of avocado.

At first, as the terminal and lateral winter buds unfold, growth appears to consist of inflorescences only. Each in-

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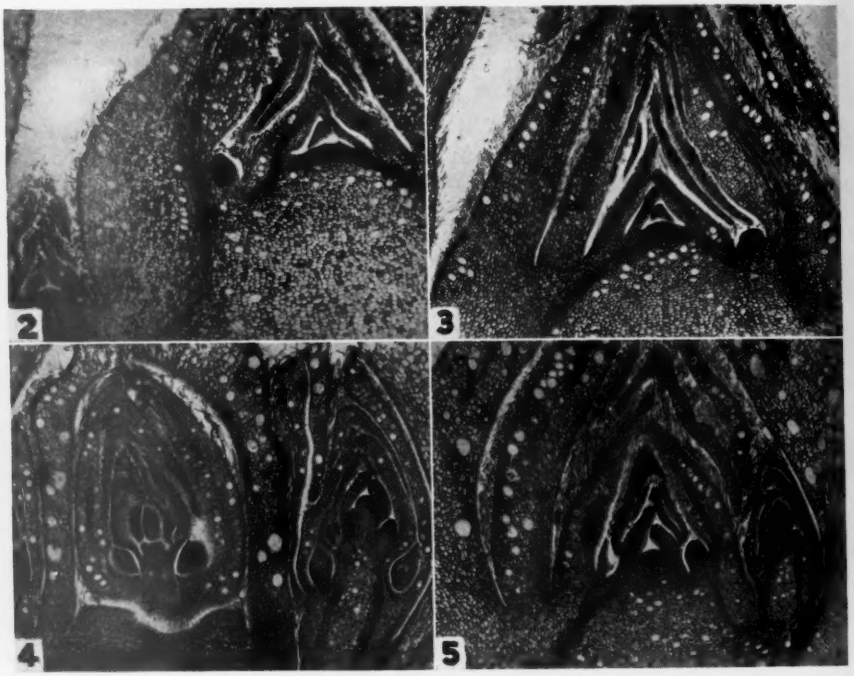
[2] ABBOT, C. E., Blossom-bud differentiation in citrus trees. Amer. Jour. Bot. 22:476–485. 1935.

florescence seems several times compound. Later it becomes evident that each main axis is an indeterminate branch. The first leaves formed on this axis are bractlike, and each subtends an axillary bud which develops into an inflorescence (fig. 1). These lateral inflorescences or secondary axes are alternately arranged on the primary indeterminate axis of the winter bud and develop acropetally, giving the whole group the appearance of a compound raceme or panicle. Subsequent development shows that actually the secondary axes constitute the inflorescences. On these axes the terminal or central flowers mature first, and the clusters are therefore cymes. The growing point of the primary axis extends the vegetative growth of the shoot beyond the region of the diverging inflorescences. Foliage leaves are formed, and the buds in their axils are vegetative.



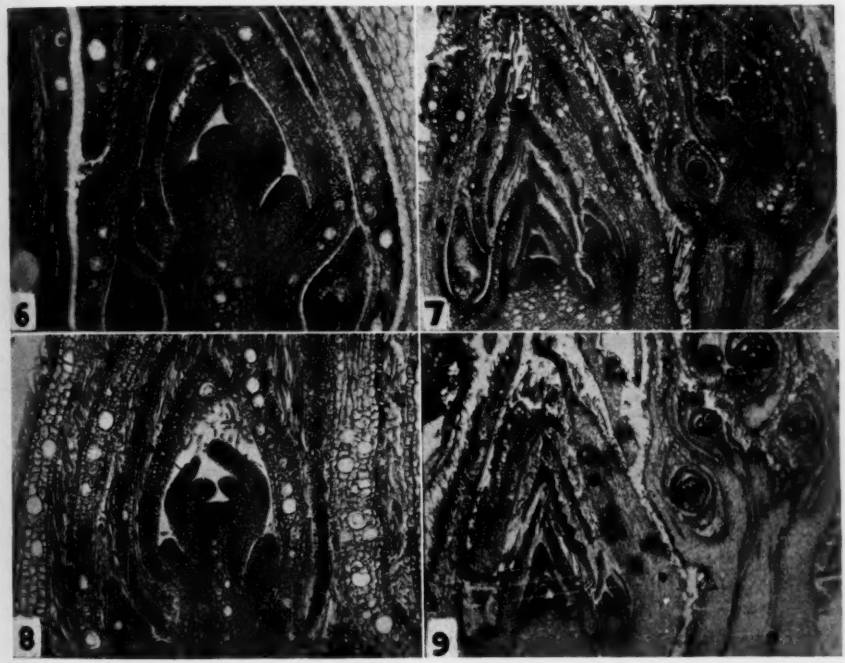
FIGS. 2–5.—Fig. 2, terminal bud of Lula variety on Oct. 8; bracts appear on lowest secondary axis. Fig. 3, terminal bud of Nabal variety on Oct. 8; meristems of secondary axes show no differentiation. Fig. 4, longisection of Nabal terminal bud, Dec. 10; secondary axes with bracts differentiated and meristems for tertiary axes apparent. (Section not truly median and therefore does not show primary apical meristem.) Fig. 5, Lula terminal bud on Dec. 10; lower secondary axis appears and differentiation is progressing. A higher secondary axis does not show differentiation. Apical meristem of primary axis appears in this section.

In mid-September most branches of the trees were actively vegetative. The first collection was made on October 8, 1940, when growth elongation had ceased and terminal buds had formed. The condition of terminal and lateral buds was found so similar in the case of each variety that this discussion will be

confined to terminal buds. In October meristematic areas were present in the axils of the bud scales. Occasionally the lowest meristem within the bud had differentiated several bracts (fig. 2); in other buds the meristems for secondary axes consisted of undifferentiated mounds of cells (fig. 3). One month later no change was recognizable in most of the buds of both varieties. One bud showed some elongation of the lowest secondary axis, but only bracts were differentiated on it. Meristematic areas in the axils of the distal bud scales were undifferentiated in all buds at this date.

From November 8 to December 10 the formation of the lower secondary axes progressed, and meristematic areas appeared in the axils of the bracts on the secondary axes (fig. 4). From the alternate arrangement of bracts and meristematic areas (fig. 6) it is obvious that this condition cannot be an early stage in the differentiation of a flower but is simply a secondary axis with bracts and axillary meristems. Whether or not a particular meristematic area would have given rise to a single flower or continued the axial ramification of the inflorescence cannot be determined. Prior to December 10 no flower primordia could be detected.

By January 13 the lateral branches



FIGS. 6–9.—Fig. 6, secondary axis of Nabal terminal bud on Dec. 10, 1940. Meristems of tertiary axes appear in axils of bracts, which are arranged alternately on secondary axis. Fig. 7, terminal bud of Lula variety of Jan. 13, 1941; lower lateral branches show early stages in flower development; higher and younger branches within bud are in various stages of development. Fig. 8, rudimentary flower terminating secondary axis in a Nabal bud, Jan. 13. Fig. 9, longisection of Lula bud collected Feb. 11; axes of orders and flowers in various stages of development.

within a terminal bud were in various stages of development, and the proximal lateral branches showed early stages in flower development (fig. 7). These branches, or inflorescences, ultimately become cymose, and the floral differentiation and anthesis of the cymules become basipetal, the middle flower opening first (fig. 1). In material collected at this time rudimentary sepals and petals were evident in the terminal flower on the secondary axis (fig. 8), and meristematic areas which would have given rise to tertiary axes appeared beneath it. A flower terminating a tertiary axis and a portion of an axis of a fourth order also appeared in this section.

Material collected February 11, 1941, showed highly complicated buds with many lateral axes of various orders and flowers in various stages of development (fig. 9). Even at this time, however, the younger distal secondary axes of the bud appeared only as meristematic mounds of cells. However, there were more well-developed axes upon the various radii than were present at progressively earlier dates.

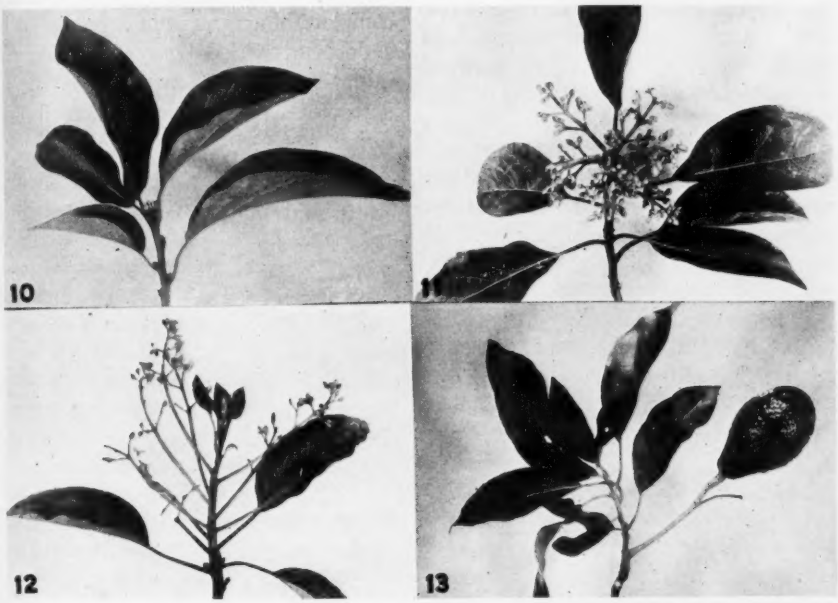
In order to compare the regional variation in the differentiation of the inflorescences of these varieties, as well as that from year to year, buds were collected bi-monthly from the trees at Orlando during the winter season of 1941–42 and monthly from trees of the same varieties on the grounds of Mrs. W. H. Krome at Homestead, Florida.

The inflorescences began their differentiation later during the winter of 1941–42 in the Orlando district than they did in 1940–41. Lula terminal buds collected December 31, 1941, were in approximately the condition shown in figure 5, which illustrates the stage reached by Lula buds at Orlando on December 10, 1940. In the Homestead region differentiation was even further delayed, and on January 14, 1942, Lula buds were still in the stage illustrated in this figure. Buds of both varieties collected the following day at Orlando showed early stages in the differentiation of flowers with the appearance of the first sepal primordia. Thus, during the period between December 31, 1941, and January 14, 1942, development took place approximately equal to that covered by the period from December 10, 1940, to January 13, 1941. Subsequent growth of the inflorescences in 1942 closely paralleled the development in the preceding year. At Orlando on February 10 Lula buds contained many flowers in which sporogenous tissue was present in the stamens, and ovule development had begun. Nabal buds were slightly less advanced and did not have sporogenous tissue. Although on January 14, 1942, buds of the Lula variety in the Homestead district were less advanced by 2 weeks than were buds at Orlando, and by a month in comparison with Orlando buds of the preceding year, by February 11 they equaled the development at Orlando. The same cannot be said of the development of the inflorescences of the Nabal avocado at Homestead. Buds collected on March 10 had developed little during the entire year. The lowest secondary axes appeared like those of the Lula variety on December 10, 1940 (fig. 5).

With the exception just mentioned, during early March the buds expanded rapidly (fig. 10), and the secondary branches from the bud underwent marked development. These secondary axes develop acropetally and give rise to tertiary branches which may either be alternate or apparently opposite in arrangement.

These tertiary branches have flowers in small clusters (cymules) in which the central blossoms opened first (fig. 11).

In the entire growth of the primary axis of the bud an indefinite number of secondary flowering axes appear and mature. Finally development of this type ceases. Nevertheless, the main apical meristem continues to grow and gives rise to the leafy or vegetative spring flush of growth (fig. 12). Each leaf on this upper portion of the main axis subtends an axillary bud which is purely vegetative in character. In a few abnormal cases the primary axis becomes determinate and a flower terminates the growth. Vegetative growth is therefore dependent upon the normally indeterminate condition of the primary axes. Although a group of inflorescences arising from a single winter bud may bear several hundred flowers, in most commercial varieties usually a single fruit matures, or—at best—a very small number. Thus most of the secondary axes or inflorescences abscise. The primary axes continue vegetative growth, and the fruits that mature are usually borne upon axes of a third or fourth order (fig. 13).



FIGS. 10–13.—Fig. 10, terminal bud just before elongation of secondary axes. Fig. 11, inflorescences expanded and ultimate cymules visible. Fig. 12, flowers and various axes abscissing and vegetative growth of indeterminate primary axis developing. Fig. 13, fruit and its relation to secondary or inflorescence axis and to primary axis of winter bud.

Summary

1. Buds of Lula and Nabal avocado collected during the winters of 1940–41 and 1941–42 at Orlando, Florida, and from the same varieties at Homestead, Florida, during the winter of 1941–42,

were studied. In these varieties floral differentiation continued over a period of months during that part of the winter when leaf formation and growth did not take place or were at a minimum.

2. Differentiation began with development of the proximal secondary axes in late October or November. There was a year-to-year variation in the time of this development for a given variety, and between varieties, as well as between trees of the same variety in different sections.

3. The first identifiable flowers appeared on the lowest inflorescences (secondary axes) in the January buds of both varieties in the Orlando district and of the Lula variety in the Homestead district; but in the Nabal variety at Homestead they had not yet appeared on March 10, 1942. Differentiation proceeded distally during the winter, and in the most distal secondary axes it was completed just prior to or during expansion of the bud. Development into complete bloom was not interrupted by a dormant period between the time of initiation and differentiation on the inflorescence and its expansion in the spring.

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OPTIMAL CONDITIONS FOR THE HYDROLYSIS OF ARABOGALACTAN BY ASPERGILLUS NIGER

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Introduction

GORTNER (16) states that, as far as is known, there is no specific enzyme by which the hydrolysis of galactans can be accomplished. The literature, however, indicates that such enzymes have been derived from various sources. BIERRY and GIAJA (3, 4) have found that the hepato-pancreatic juice of *Helix pomatia* attacks both mannans and galactans, and that the intestinal juice of *Astacus fluviatilis* sets free the galactose of the mannogalactan of alfalfa; the mannose, however, is only partially freed and in a degree is dependent upon the concentration of the enzyme. HERRISEY (17) has shown that certain species of *Aspergillus* are able to produce enzymes hydrolyzing mannans and galactans, when grown on solutions containing the respective carbohydrates. LUTZ (26, 27) has found that many Hymenomycetes secrete enzymes capable of hydrolyzing hemicelluloses and gums. He obtained the decomposition by enzymes of hemicelluloses—arabans, mannans, and galactans—which occur in many plants. In studying the aerobic decomposition of hemicelluloses, NORMAN (33) employed some seventy molds, mostly *Aspergilli* and *Penicillia*, and found that all the fungi tested grew reasonably well on media containing the crude hemicellulose from oat straw, which included 8.2 per cent uronic acid, 50.8 per cent pentose, and the remaining 41 per cent presumably hexosans. The majority of the molds grew at least as well as on glucose and a number better than on that sugar. In some cases there was a production of a cleared zone around the colony, due to an exo-enzyme. He believed it likely that the ability to ferment hemicelluloses will be demonstrated a general property of the common fungi. WAKSMAN and DIEHM (53) found that *A. niger* was able to decompose galactans to the extent of 37 per cent in 42 days but noted that the galactan was more resistant to decomposition by microörganisms than were mannans and xylans. The galactan used by these investigators was obtained from various sources, including straws, which were classified by them as hemicelluloses.

To study the action of a galactanase, if such exists, a source of the enzyme is required. Because *A. niger* seems able to produce a wide variety of enzymes, one of its strains was chosen for this study. Another requirement is a suitable galactan, which was supplied by arabogalactan.

Arabogalactan is a polysaccharide obtained from western larch (*Larix occidentalis*) in quantities varying from 10 to 20 per cent of the heartwood. It is also present to some extent in other species of larch and in southern pine. It can be extracted readily from sawdust by simple water leaching and consequently should undergo little chemical change during its isolation. It is fairly well characterized as a compound containing arabinose and galactose in the ratio of one molecule to six, respectively. Although the

exact arrangement of the sugar residues in the molecule is still unknown, information on that point is accumulating (56, 57, 58), so that before long not only the type and location of the linkages between the sugar residues, but also the spatial relationship between them, should be known. This substrate should offer possibilities for fundamental work on the action and specificity of many enzymes.

HOSSFELD (20) has investigated the action of several strains of *A. niger* on water extracts of larch sawdust, without obtaining conclusive results. Preliminary unpublished work by OWENS indicated that purified arabogalactan could be used as a satisfactory substrate for such molds. The optimal conditions for the maximum hydrolysis of arabogalactan by *A. niger* have now been established and are reported in this paper.

Material and methods

Arabogalactan was prepared in the same manner as reported earlier (34), by extracting sawdust from the heartwood of a western larch stump with water. The extract was decolorized with charcoal and the polysaccharide precipitated from solution by ethyl alcohol. The fraction obtained by addition of from one to four volumes of alcohol was used. The first fraction usually contained some colored impurities and inorganic matter. The remaining colored impurities were removed with charcoal. The fraction obtained with more than four volumes of alcohol was small in amount and was not economical to separate. Arabogalactan prepared in this manner analyzed 80–85 per cent anhydro-galactose and had an ash content of 0.1 per cent. The viscosity-concentration coefficient (10) was 5.8×10^{-3}, obtained by use of water solutions containing 1–4 per cent arabogalactan.

The strain of *Aspergillus niger* used was Thom 3528.7, obtained by Dr. John Ehrlich of the School of Forestry, University of Idaho, from Dr. Charles Thom of the Division of Soil Microbiology, Bureau of Plant Industry, United States Department of Agriculture.

All the chemicals in the media were c.p. and were used without further purification. The basal nutrient was made up within the limits proposed by CURRIE (7) and contained 1.0 gm. KH_2PO_4, 0.15 gm. $MgSO_4$, and 2.5 gm. NH_4NO_3 in one liter of solution. The pH was adjusted to 3.5 with HCl and measured by means of a Beckman glass electrode.

To analyze for reducing sugars, the method of FOLIN and WU (11) was used. The standard was a solution of galactose, so that the results are expressed in terms of galactose, but it must not be assumed that all or any of the reducing sugar is in that form.

Two methods of preparation of inoculum were employed. When the organism was first obtained, transfers were made to Czapek's agar, using the stab transfer method. These cultures were allowed to grow at room temperature for a few days and then were placed in a refrigerator maintained at approximately 10° C. Prior to trials, one tube was removed from the refrigerator and allowed to stand at room temperature overnight, and inoculations were made directly from this stock culture. In the second method an "activated" culture was used. The activation consisted in first transferring the organism from the stock culture to a flask containing 25 ml. of basal nutrient and 2.5 gm. of arabogalactan. The mold was allowed to grow for 10 days at 28° C. before using it as a source of inoculum.

Inoculations were made from either source to 25 ml. of basal nutrient con-

taining 10 per cent arabogalactan. In all experiments 250-ml. titration flasks were used, stoppered with rolled plugs of nonabsorbent cotton. The flask and contents were sterilized in a steam-pressure cooker at 15 pounds per square inch for 20 minutes, and inoculations were made in a sterilized transfer cabinet.

At the end of each period of investigation the mold cultures were killed by the addition of 10 ml. of a 0.1 per cent mercuric chloride solution saturated with thymol. The mycelium was filtered off, washed with distilled water until the total volume of the filtrate and washings was about 100 ml., and then each solution was brought to volume in 100-ml. volumetric flasks. Aliquot portions of these solutions were used for analyses. If mold weights were desired, the mycelium was filtered on a weighed sintered glass crucible and dried to constant weight at 70° C.

Controls were run in all series. All pH values were checked with a glass electrode.

Results

Effect of pH

To determine the effect of variation in pH, a series of flasks was set up so that the pH varied one-half a unit from pH 2.0 to 5.0. The temperature of these runs was 35° ± 0.5° C. The reducing value was determined at the end of 1, 3, 5, 8, 12, and 16 days. A maximum reducing value of 6.2 mg. of galactose per gram of arabogalactan in the original substrate was obtained at a pH of 3.5 in 5 days. Other maxima were: at pH 2.0, 2.9 in 1 day; at pH 2.5, −8.5 in 16 days; at pH 3.0, −6.2 in 16 days; at pH 4.0, 2.8 in 5 days; at pH 4.5, 5.2 in 3 days; and at pH 5.0, −0.4 in 1 day. The decrease in reducing values at low pH values resulted from the fact that there was some acid hydrolysis of arabogalactan during sterilization, so that the mold grew on the liberated galactose or other degraded sugar residue. When the experimental value was subtracted from the control value, a negative number resulted. The negative value at pH 5.0 merely reflected the poor growth of the mold at that pH and was barely beyond the limits of precision of the method.

Effect of temperature

In this series the pH value was kept constant at 3.5 and the temperature was varied. Flasks were removed from the constant temperature airbath for analysis at the end of 3, 5, 8, and 12 days. A maximum reducing value of 12.6 gm. of galactose per gram of arabogalactan was obtained at 28° C. in 8 days. A value nearly as great, 12.0, was obtained at 30° in the same period of time. Analysis of the growth curves in each case indicates that there is little to choose between these two temperatures. At 25° C. the maximum of 9.4 was obtained in 8 days; at 35° C. the maximum of 6.2 was obtained in 5 days.

Effect of method of inoculum preparation

With the temperature at 28° C. and the pH at 3.5 the maximum reducing value obtained with the stock culture was 12.6 in 8 days, while the maximum reducing value with the activated culture was 7.9 in 8 days. This would indicate that successive transfers from a culture of the mold grown on arabogalactan were not beneficial, differing from Funke's (14) finding with diastase produced by *A. niger*.

Effect of heavy metals

Heavy metals, particularly zinc, manganese, molybdenum, copper, gallium,

and iron, have been reported effective in increasing the rate of growth of *A. niger* (5, 12, 15, 22, 29, 35, 47, 48, 49, 52, 59). When the temperature was kept constant at 28° C. and the pH at 3.5, the data in table 1 were obtained at 4 and 6 days. Judging from the values given in the table, all the heavy metals mentioned (except gallium) are effective in increasing the rate of growth of the tested strain when it is grown on arabogalactan. Except for gallium and molybdenum, no data on the individual heavy metals have been obtained under these conditions.

TABLE 1

EFFECT OF HEAVY METALS ON GROWTH OF A. NIGER GROWN ON ARABOGALACTAN

BASAL NUTRIENT	GROWTH (MG. MYCELIUM)		REDUCING VALUE (MG. GALACTOSE/GM. ARABOGALACTAN)	
	4 DAYS	6 DAYS	4 DAYS	6 DAYS
No supplement	4*	6*	2.6*	3.1*
Supplemented with Fe, Cu, Mn, Zn†	10*	15*	4.0*	8.6*
Supplemented with Fe, Cu, Mn, Zn, Mo†	20	58	.....	.....
Supplemented with Fe, Cu, Mn, Zn, Mo, Ga†	19	.....	.....	.....

* Interpolated from growth curve.

† Per liter of nutrient: Fe 0.2 mg., Cu 0.04 mg., Mn 0.02 mg., Zn 0.14 mg., Mo 0.02 mg., Ga 0.02 mg.

EFFECT OF YEAST

Yeast has been recognized as a growth stimulant for molds by many investigators, although there seems to be some disagreement about the actual part it plays in causing increase in growth (13, 21, 48, 50, 51). Considering the success that others had experienced, it was thought desirable to include experiments with yeast extract in the nutrient. The amount used was that obtained from 250 mg. of yeast[1] per 25 ml. of nutrient. The extract represented 95 mg. of solid matter, or 38 per cent of the total yeast.

When the pH was kept at 3.5 and the temperature at 28° C., yeast caused the mycelium to reach a weight of 183 mg. in 4 days, compared with only 20 mg. obtained with the control. Similar striking results were obtained when the nutrient contained glucose instead of arabogalactan. With glucose the control mycelium weighed 85 mg. after 44 hours, while the mycelium grown in presence of the yeast extract weighed 508 mg. Yeast evidently contains a factor or factors which stimulate the growth of *A. niger*, and this stimulation is not dependent upon the presence of some esoteric carbohydrate, such as arabogalactan, but is effective even with glucose, which is considered a satisfactory growth material when supplemented by the proper minerals.

Although it was not the purpose of this study to investigate the nature of this stimulant, a few experiments along that line were tried. The results showed that the stimulants were soluble in 70 per cent alcohol, and that they were not adsorbed completely on Lloyd's reagent from acid or alkaline solutions. A partial adsorption was obtained at pH 1.5 and 5.0. The factor(s) were completely adsorbed on charcoal but could not be eluted readily by use of acetone and ammonia, as recommended by ROBBINS (36) for his Z factor. The incomplete adsorption on Lloyd's reagent indicates that the factor is not folic acid (28), which is adsorbed on that material. Whether it is the new factor reported by SNELL and PETERSON (44) is questionable, since STOKSTAD (51) has offered

[1] Northwestern Yeast Company, Chicago, Illinois.

TABLE 2

VARIATIONS IN GROWTH RATE OF A. NIGER ON SUPPLEMENTED MEDIA CONTAINING ARABOGALACTAN. TIME OF INCUBATION 4 DAYS UNLESS OTHERWISE INDICATED. RESULTS ARE AVERAGES OF DUPLICATE CULTURES

SUPPLEMENT TO BASAL NUTRIENT	CONCENTRATION OF SUPPLEMENT (MG./25 ML.)	MYCELIUM WEIGHT MINUS CONTROL (MG.)
1. Thiamin	0.2	8*
2. Thiamin	0.4	− 1*
3. Riboflavin	0.05	− 11
4. Riboflavin	0.2	− 22*
5. Nicotinic acid	0.05	− 11
6. Nicotinic acid	0.1	− 6
7. Nicotinic acid	0.2	− 8*
8. Calcium pantothenate	0.025	34*
9. Calcium pantothenate	0.05	− 3
10. Calcium pantothenate	0.1	− 7
11. Pyridoxine	0.025	− 5
12. Pyridoxine	0.05	− 2
13. Pyridoxine	0.1	43*
14. Ascorbic acid	1.0	− 14*
15. Ascorbic acid	2.0	− 17*
16. Asparagine	125.0	− 15
17. Asparagine	250.0	− 13
18. Pyruvic acid	0.5	− 7
19. Pyruvic acid	1.0	− 3
20. Glycolic acid	0.15	− 6
21. Glycolic acid	0.30	− 7
22. Glutamic acid	0.25	− 19*
23. Glutamic acid	0.50	− 21*
24. Pyruvic acid	0.5	.....
Glycolic acid	0.15	− 14*
25. Thiamin	0.2	.....
Pyruvic acid	0.5	− 3
Pyridoxine	0.1	.....
26. Thiamin	0.2	.....
Calcium pantothenate	0.025	− 3
Pyridoxine	0.1	.....
27. Ascorbic acid	4.0	.....
Pyruvic acid	0.5	15
Glycolic acid	0.15	.....
28. Thiamin	0.2	.....
Riboflavin	0.05	.....
Nicotinic acid	0.1	− 7
Calcium pantothenate	0.025	.....
Pyridoxine	0.05	.....
29. Biotin	0.0001	0
30. Biotin	0.0003	− 3
31. Acid-hydrolyzed casein	10.0	10
32. Acid-hydrolyzed casein	20.0	6
33. Yeast extract	95.0	163

* Six-day growth.

evidence to show that it is a nucleotide, and our incomplete results indicate that the active factor(s) in the present experiments are not nucleotides.

EFFECT OF OTHER GROWTH STIMULANTS

Several experiments were run in an effort to determine whether the growth factors in yeast were known members of the vitamin B complex, mixtures of amino acids, or other growth stimulants. For these experiments the usual nutrient and heavy metal supplement were used at pH 3.5 and temperature of 28° C. The materials to be tested were added at the concentration levels noted in tables 2 and 3 and were chosen on the basis of their known presence in yeast or because they

TABLE 3

INFLUENCE OF YEAST AND CASEIN ON GROWTH OF A. NIGER USING GLUCOSE AS SOURCE OF CARBON. TIME OF INCUBATION 44 HOURS. INOCULATED FROM STOCK. RESULTS ARE AVERAGES OF TRIPLICATE CULTURES

SUPPLEMENT TO BASAL NUTRIENT	QUANTITY ADDED (MG./25 ML.)	MYCELIUM WEIGHT MINUS CONTROL (MG.)
1. Casein	10	.....
Cystine	5	19
2. Casein	10	.....
Cystine	5	23
Vitamins*	0*	.....
3. Yeast extract	9.5	135
4. Casein	100	.....
Cystine	5	203
5. Casein	100	.....
Cystine	5	161
Vitamins*	0*	.....
6. Casein	80	.....
Cystine	5	234
Yeast nucleic acid	10	.....
Inositol	10	.....
7. Yeast extract	95	423
8. p-aminobenzoic acid	0.00025	2
9. p-aminobenzoic acid	0.0025	7
10. p-aminobenzoic acid	0.025	3

* Vitamins added per 25 ml. were: thiamin 200 μ gm.; calcium pantothenate 25 μ gm.; pyridoxine 100 μ gm.

had been used successfully by others (1, 2, 6, 9, 18, 23, 24, 25, 30, 31, 32, 37, 38, 39, 40, 41, 42, 43, 45, 46, 50, 54). None of the known water-soluble vitamins, including biotin, p-aminobenzoic acid, and inositol, caused significant increase in the growth rate of the mold. The amino-acid mixture obtained from the acid hydrolysis of casein did increase the growth rate, but the increase was well below that noted with yeast extract. This gives ample evidence that yeast supplies some agent not yet known as a growth factor for *A. niger*.

Effect of source of arabogalactan

All this work was done using arabogalactan obtained either from an old larch stump or from the butt log of a larch tree 25 cm. in diameter. The viscosity-concentration coefficient of the arabogalactan from either source was the same, 5.8×10^{-3}, and the biological activity as measured by growth of *A. niger* seemed the same. A third sample of arabogalactan taken from a tree about 95 cm. in diameter had a viscosity-concentration coefficient of 5.1×10^{-3}. It permitted somewhat more rapid growth of the mold. Controlled experiments using the last of the older samples of arabogalactan gave mycelial weights of 187 mg. in 6 days, while the less viscous arabogalactan permitted the mold to grow to 273 mg. A fourth sample of arabogalactan collected from another large tree permitted growth of 270 mg. of mycelium in the same experiment. The last two samples showed the greatest difference in viscosity effect, yet have about the same effect on the growth rate of the mold. The earlier samples, with an intermediate viscosity effect, do not support nearly such vigorous growth.

The reason for this is not yet apparent. It is not related to viscosity characteristics or to season, because the third sample was collected from a tree cut in June, 1941, and the fourth sample was obtained from a tree cut in October, 1941. The data are still too few to justify a fair conclusion, however.

Effect of metabolites

The mold during growth on arabogalactan will produce some metabolites different from those developed when the mold is grown on sucrose. One of these, galactose, is believed to be a poor source of carbon for molds (8, 19, 55). From similar experiments Waterman (54) ranks galactose and galactans with poisons such as copper sulphate, and narcotics like salicylic acid, in causing decreased growth, loss of color, and decreased sporulation.

One series of experiments involved the comparison of mycelial development on the ordinary nutrient with 10 per cent arabogalactan, heavy metals, and yeast for the controls and a similar medium containing 0.2 per cent galactose in addition. This concentration of galactose was arrived at by assuming that the reducing values found were due to galactose. Although this assumption is false, it gives the maximum possible concentration of that sugar likely to be found in the media. The mycelial weights averaged 428 mg. in the first case and 500 mg. in the second, showing that the toxic effect of galactose was not a problem in these experiments.

In the absence of yeast, however, it has been found that a second mycelial mat generally does not develop on the same nutrient after one mat has been removed, indicating that some toxic metabolic product is formed by the mold. No information is available on the nature of this product.

In the presence of the yeast extract, however, a second mat can be grown in about the same time as required for the first one. Evidently one of the functions of the yeast extract is either to combine with the toxic material in some way or to prevent its formation.

Discussion

Whether *A. niger* develops a specific galactanase when grown on arabogalactan has not been determined, but the optimum conditions for the growth of the mold have been established, and work is now possible on the concentration of the enzyme which hydrolyzes the galactan.

Except for the differences in the weights of the mycelia, the results obtained with *A. niger* grown on arabogalactan are very similar to those obtained by STEINBERG (48–50), using glucose or sucrose in the substrate. He has reported gallium as a necessary element, which disagrees with the results reported here; but no attempt was made to exclude gallium, and it might have been present in small quantities in some of the c.p. salts used in the nutrient.

Yeast extract contains some agent or agents which stimulate the growth of *A. niger* in much the same way that riboflavin stimulates the growth of *Lactobacillus casei*. There is some evidence that yeast either prevents the formation of or combines with toxic metabolic products of the mold grown on arabogalactan.

It has been shown that the water-soluble material in yeast differs from any of the known vitamins of the B complex or from growth stimulants ordinarily associated with molds. Evidently the strain of *A. niger* used in this work is capable of synthesizing many of the vitamins recommended for other strains. It cannot be considered that this stimulating effect of yeast is due to amino acids or nucleic acid, inasmuch as a casein hydrolysate or nucleic acid with or without vitamins was much less effective than yeast extract at the same concentration of total solids.

Little can be said at this time regarding the poor growth of the mold on arabogalactan. Since the molecule is either spherical or slightly ellipsoidal, the galactosidic linkages may be folded into the structure in such a way that they cannot readily make contact with the enzyme, which would prevent catalysis. On the other hand, the galactosidic linkages themselves may offer the steric hindrance preventing proper contact between the substrate and enzyme, in the same way that the branching of glucosidic chains in starch hinders the activity of certain amylases. The slow rate of hydrolysis and the biological variability of arabogalactan warrant further investigation.

Summary

1. The optimum conditions for growth of *Aspergillus niger* (Thom 3528.7) on a substrate containing arabogalactan, CURRIE'S salt medium supplemented with salts of iron, zinc, copper, manganese, and molybdenum, and a yeast extract are a pH of 3.5 and a temperature between 28° and 30° C.

2. *Aspergillus niger* produces an enzyme capable of hydrolyzing arabogalactan; whether or not this is a specific galactanase has not been determined.

3. There is a growth factor or factors not yet identified in yeast.

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TREATMENT OF SEEDS WITH SYNTHETIC GROWTH-REGULATING SUBSTANCES

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(WITH TWO FIGURES)

Introduction

It has been suggested that increased growth of plants may be obtained by treating the seeds with growth regulators. Results of some experiments seem to support this contention (**1, 2, 3, 5, 8, 10, 11, 13, 14, 17, 20**), but other investigations have failed to give corroboration of such results (**4, 6, 7, 9, 12, 15, 16, 18, 19**). This confusion may be due in part to differences in the environmental factors under which the various investigations were made. Until enough data are available from varied localities, it is not possible to estimate conclusively the merits of treating seeds with growth-regulating substances, and much additional evidence is still required.

The experiments reported here concern the effects of treatment of seeds with a number of synthetic growth-regulating substances and combinations of them over a wide range of concentrations.

Methods

These experiments were conducted at the U.S. Bureau of Plant Industry Station, Beltsville, Maryland; at the University of Chicago; and at Lake Geneva, Wisconsin. Growth-regulating substances were applied either by dusting the seeds with the substance dispersed in talc dust or by soaking them in water solutions of the substances.

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GREENHOUSE EXPERIMENTS

Greenhouse experiments were conducted at Chicago and at Beltsville. To permit a survey of the effects of various growth-regulating substances, attention was confined at Chicago to oat plants and at Beltsville to radish. These experiments were carried out from January to June, 1942. The experimental design, treatments, and materials used were similar at the two localities.

Preparations of the substances in talc were made by dissolving the substance in 50 cc. of 95 per cent ethyl alcohol and adding this to 50 gm. of powdered talc, forming a thin paste. The alcohol was then evaporated off in a drying oven at 80° C. and the dried cake finely pulverized. Water solutions of the substances were prepared by dissolving them directly in water. Those that did not dissolve easily were first dissolved in a few cubic centimeters of ethyl alcohol and then added to the water. Except where noted, the seeds were soaked for 14 hours in these solutions.

A randomized block statistical design was used. With radish each block was replicated seven times over benches which extended through the middle of the greenhouse. The experiments with oat were usually replicated ten times. Across each end of each bench was a "guard" row of plants grown from untreated seeds. Each block consisted of nine treatments of six pots each across the bench. There were four seeds per pot

in the case of radish and five seeds in the case of the oat, making a total of 1512 seeds in each experiment with radish and 2700 seeds in each experiment with oat.

Six-inch clay pots filled with a potting mixture containing five parts loam, two parts sand, and one part manure, were used with radish. The pH of the mixture was 7.0. After planting, the seeds were firmed in and covered with a light top dressing of one part sand and one part loam. The oat grains were planted in a similar potting mixture in 1½-liter glazed crocks. A ¼-inch top dressing of pure quartz sand was used. Both the oat and radish were watered whenever required. For radish the greenhouse temperature was maintained as nearly as possible at 60°–65° F. for the night and at 70°–75° F. in the daytime. For oat it was 55°–60° F. at night and 70°–75° F. in the daytime.

In one greenhouse experiment radish seeds were treated with commercial preparations of growth-regulating substances and were grown in three types of soil: a sandy loam (pH 5.5), a potting mixture (pH 7.0), and a muck mixture (pH 5.2). Untreated radish seeds were also grown in these different soil mixtures but were watered every 4 days with vitamin B_1 at a concentration of 0.01 mg. per liter in one series and 8.00 mg. per liter in another.

The radish plants were usually grown for 28 days before harvesting. In three experiments this was exceeded by a day or so, while in a few others the plants were harvested before this time, but all the plants in any one experiment were harvested on the same day. Germination counts were made 1 week after planting.

The oat plants were grown about 42 days before harvesting. Germination counts were made 14 days after planting.

At harvest, the fresh weight of the tops and roots of radish was measured to the nearest ±0.5 gm., the roots having been previously washed free from soil. The tops were then placed directly into a forced-draft electric drying oven at 80° C. The roots were sliced in thin sections, spread out on paper, and similarly dried. After both the tops and roots were brittle dry, about 24 hours, they were taken from the oven and allowed to cool to room temperature. They were then weighed to within ±0.05 gm. on a torsion balance.

The oat tops were cut from the roots at ground level. Height and fresh weight measurements were taken. The tops were then dried and weighed similarly.

Field experiments

All the field experiments were performed at Beltsville, from April to September, 1942, except for duplicate plantings of radish and sugar beet at Lake Geneva, Wisconsin.

The soil used at Beltsville is a light sandy clay loam, with pH 5.3. The soil has been in cultivation for several years and prior to the spring plowing received one application of rotted manure. At planting time the 6-acre area was fertilized with a 5–8–5 artificial fertilizer at about 300 lb. per acre. The soil was prepared and crops planted according to usual farm practice.

Buckwheat, corn, oat (var. Fulghum), and Zuchini squash were planted in Latin squares for statistical analysis. The other crops were planted in rows as randomized blocks, with a minimum of eight replications. During a spring drought irrigation was necessary for 3 weeks.

The radish and sugar beet seeds grown at Lake Geneva were treated with the same growth-substance preparations used at Beltsville and were grown in rich

loam soil which had a pH of 7.3. They were planted in rows as randomized blocks, each replicated six times. The cross checked. After germination each hill was thinned to three plants. The field was cross cultivated.

TABLE 1

AVERAGE DRY WEIGHT IN GRAMS OF RADISH PLANTS FROM SEEDS DUSTED WITH GROWTH-REGULATING SUBSTANCES IN TALC. GERMINATION AT LEAST 90 % IN ALL CASES. EACH FIGURE AVERAGES ABOUT 150 PLANTS

EXP. NO.	GROWTH-REGULATING SUBSTANCE	SEEDS NOT DUSTED	SEEDS DUSTED (AS P.P.M. IN TALC)						
			0	2	20	200	1000	10,000	30,000
1.	Indolebutyric acid	0.729	0.832	0.683	0.693	0.714	.....	.....	.....
9.	Indolebutyric acid	.657	.741	.....	.....	.....	0.769	0.738	0.741
2.	Naphthalene acetamide	.773	.776	.753	.770	.794	.....	.....	.....
9.	Naphthalene acetamide	.657	.741	.....	.....	.....	.831	.734	.729
3.	Napthoxyacetic acid	.574	.577	.564	.561	.574	.....	.....	.....
10.	Napthoxyacetic acid	.614	.638	.....	.....	.....	.631	.702	.636
4.	Phenylacetic acid	.763	.752	.791	.749	.757	.....	.....	.....
6.	Phenylacetic acid	.613	.600	.....	.....	.....	.585	.578	.590
5.	Naphthaleneacetic acid	.854	.885	0.892	0.905	0.848	.....	.....	.....
6.	Naphthaleneacetic acid	0.613	0.600	.....	.....	.....	0.555	0.458	0.460

TABLE 2

AVERAGE DRY WEIGHT IN GRAMS* OF RADISH PLANTS FROM SEEDS SOAKED 14 HOURS IN WATER SOLUTIONS OF GROWTH-REGULATING SUBSTANCES

EXP. NO.	GROWTH-REGULATING SUBSTANCES	SEEDS NOT SOAKED		SEEDS SOAKED (AS P.P.M.)							
				0		10		100		200	
		WEIGHT (GM.)	GERMINATION (%)	WEIGHT (GM.)	GERMINATION (%)	WEIGHT (GM.)	GERMINATION (%)	WEIGHT (GM.)	GERMINATION (%)	WEIGHT (GM.)	GERMINATION (%)
1.	Indolebutyric acid	0.729	96.4	0.726	97.6	0.723	90.5	0.598	88.1	.....	.....
2.	Naphthalene acetamide	.773	97.0	.832	92.2	.786	89.3	.581	96.4	.....	.....
3.	Naphthoxyacetic acid	.574	92.8	.570	91.7	.533	95.2	.278	80.9	.....	.....
4.	Phenylacetic acid	.763	97.0	.765	94.0	.749	91.7	.750	86.3	0.724	90.5
5.	Naphthaleneacetic acid	0.854	92.3	0.922	91.1	0.793	87.5	0.424	81.5	0.160	47.6

* Average dry weight = $\frac{\text{Total dry weight}}{\text{Germination percentage} \times 168}$.

yields of all the farm crops except corn and sugar beet were weighed to within ± 50 gm.

The forty-nine individual corn plots within a square, each consisted of twenty hills. The hills were 3 feet apart and were

Two of the four Latin squares of corn received the usual application of manure as well as an application of about 400 lb. per acre of 5–8–5 fertilizer after plowing. At planting, another application of the fertilizer was made at each hill. The oth-

er two squares received no manure or fertilizer.

The fresh weight of the top growth of the corn was determined to within ±250 gm. on a large platform balance. The dry weight was determined from a quart sample of chopped material (fodder) from five plants out of each plot. It was dried at 80° C. for 96 hours in a forced-draft electric drying oven.

TABLE 3

GROWTH OF RADISH PLANTS WATERED WITH VITAMIN B_1 OR GROWN FROM SEEDS DUSTED WITH COMMERCIAL PREPARATIONS OF GROWTH-REGULATING SUBSTANCES

Exp. no.	Seed treatment	Sandy soil loam		Potting soil mixture		Muck soil mixture	
		Germination (%)	Average dry weight* (gm.)	Germination (%)	Average dry weight (gm.)	Germination (%)	Average dry weight (gm.)
7.	a. Untreated	90.4	0.370	87.5	0.720	96.4	1.116
	b. Commercial preparation A	90.5	.437	95.2	.736	93.4	1.090
	c. Commercial preparation B	92.9	.374	92.9	.645	94.6	1.127
	d. Watered seven times with 0.01 mg./l. vitamin B_1	80.4	.364	91.1	.784	94.0	1.064
	e. Watered seven times with 8.00 mg./l. vitamin B_1	86.3	0.353	90.5	.670	92.3	1.086
9.	a. Untreated	.....	.....	91.2	.657	.....	.....
	b. Commercial preparation C	.....	.....	92.8	0.749	.....	.....

* Average dry weight = $\frac{\text{Total dry weight}}{\text{Germination percentage} \times 168}$.

Results

Greenhouse experiments

With radish there were no statistically significant increases in either fresh or dry weight of the tops or roots of plants from any of the treated seeds over those from untreated (tables 1–4). When the seeds were soaked 14 hours in a water solution containing 500 p.p.m. indolebutyric acid, strong inhibition of growth was noted (fig. 1). Inhibition of growth is indicated with naphthaleneacetic acid at 10 p.p.m. (table 2).

The only response of radish that might be interpreted as increased growth was from soaking the seeds 14 hours in a 100 p.p.m. solution of indolebutyric acid. After 9 days the hypocotyls of the plants from treated seeds were 130 per cent longer than those of plants from untreated seeds (fig. 2), a statistically significant difference at the 1 per cent level. At harvest time, however, the dry weight of the plants with the longer hypocotyls was actually less than that from untreated seeds. Likewise at harvest time the hypocotyls of the plants from the treated seeds were not significantly longer than those from the untreated. This was probably owing to the fact that the plants with the longer hypocotyls were procumbent on the top of the soil and so did not grow as vigorously as did the more erect plants or develop the bulbous swelling characteristic of radish.

As with radish, the treatment of oat grains with growth regulators did not increase their subsequent growth over plants from untreated seeds (tables 5–7).

TABLE 4

GROWTH OF RADISH PLANTS FROM SEEDS TREATED WITH LEVULINIC ACID

EXP. NO.	SEED TREATMENT	GERMINATION (%)	AVERAGE DRY WEIGHT* (GM.)
8.	a. Untreated	95.8	0.547
	b. Dusted with talc dust only	92.8	.550
	c. Dusted with talc dust 1000 p.p.m. levulinic acid	94.0	.558
	d. Same as c, 10,000 p.p.m.	93.4	.579
	e. Same as c, 100,000 p.p.m.	93.4	.527
	f. Soaked 4 hours in water only	92.8	.611
	g. Soaked 4 hours in 1000 p.p.m.†	83.9	.607
10.	a. Untreated	93.6	.614
	b. Soaked 4 hours in water only	92.1	.670
	c. Soaked 4 hours in 500 p.p.m. levulinic acid in water	87.7	.767
	d. Same as c, but leaves sprayed at six intervals with 0.1% levulinic acid in water	90.1	.710
	e. Soaked 4 hours in 1000 p.p.m. levulinic acid in water, and leaves sprayed as in d	82.9	0.710

* Average dry weight = $\frac{\text{Total dry weight}}{\text{Germination percentage} \times 168}$.

† Soaking 4 hours in 10,000 p.p.m. levulinic acid resulted in no germination.

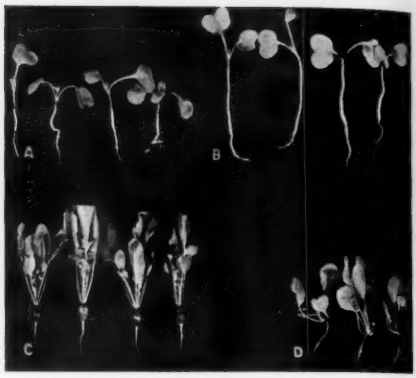

FIG. 1.—Radish plants from seeds soaked in indolebutyric-acid solutions: *A*, plants 11 days old; seeds soaked in water for 14 hours. *B*, same but soaked in 100 p.p.m. indolebutyric-acid solution. *C*, plants 28 days old; seeds soaked in water for 14 hours. *D*, same as *C* but soaked in 500 p.p.m. indolebutyric-acid solution.

FIG. 2.—Radish plants from untreated seeds after 28 days, showing effect of soil on growth. Grown on: *A*, sandy clay loam soil; *B*, potting soil mixture; *C*, muck soil mixture.

Table 5 shows that although there was no difference due to the treatment, the time of year was very influential in the total growth of the tops. Plants grown in February averaged 0.064 gm. per plant and were about 25 cm. tall after 42 days, per cent. Application of levulinic acid as a spray in conjunction with treatment was ineffective in increasing growth (table 7).

Commercial preparations for seed treatment applied as dust according to

TABLE 5

GROWTH OF OAT PLANTS, AS DRY WEIGHT OF TOPS IN GRAMS, FROM SEEDS DUSTED WITH GROWTH-REGULATING SUBSTANCES. EACH FIGURE AVERAGES ABOUT 260 PLANTS. GERMINATION 80–90% IN ALL TREATMENTS

Exp. no.	Growth-regulating substance	Period of growth	Seeds not dusted	Seeds dusted (as p.p.m. in talc)						
				0	2	20	200	1000	10,000	33,000
1.	Naphthaleneacetic acid	Feb.–Mar.	0.064	0.060	0.060	0.065	0.062	.....	.....	.....
6.	Naphthaleneacetic acid	Apr.–May	.141	.140	.....	.....	.....	0.147	0.134	0.124
3.	Phenylacetic acid	Feb.–Mar.	.107	.109	.106	.107	.104	.....	.....	.....
7.	Phenylacetic acid	Apr.–May	.140	.135	.....	.....	.138	.142	.142	.135
4.	Indoleacetic acid	Mar.–Apr.	.119	.111	0.116	0.111	0.119	.....	.....	.....
6.	Indoleacetic acid	Apr.–May	.141	.140	.....	.....	.....	.134	.140	.141
8.	Indolebutyric acid	Apr.–May	.162	.167	.....	.....	.....	.163	.170	.156
10.	Naphthalene acetamide	May–June	0.234	0.217	.....	.....	.....	0.228	0.185	0.174

TABLE 6

GROWTH OF OAT PLANTS, AS DRY WEIGHT OF TOPS IN GRAMS, FROM SEEDS SOAKED 14 HOURS IN WATER SOLUTIONS OF GROWTH-REGULATING SUBSTANCES. EACH FIGURE AVERAGES ABOUT 270 PLANTS. GERMINATION 85–90% IN ALL TREATMENTS

Exp. no.	Growth-regulating substance	Period of growth	Seeds not soaked	Seeds soaked (as p.p.m.)			
				0	10	100	500
1.	Naphthaleneacetic acid	Feb.–Mar.	0.0642	0.0658	0.0627	0.0641	0.0513
3.	Phenylacetic acid	March	.1076	.1062	.1066	.1084	.1040
4.	Indoleacetic acid	Mar.–Apr.	.1192	.1178	.1179	.1186	0.1234
5.	Indolebutyric acid	Apr.–May	.1620	.1780	.1720	.1680	.....
6.	Naphthalene acetamide	Apr.–May	0.2340	0.2040	0.2260	0.2170	.....

while those grown in May and June weighed 0.234 gm. and averaged 35 cm. Soaking seeds in solutions of naphthaleneacetic acid stronger than 500 p.p.m. resulted in decided decrease in germination.

Soaking oat grains in a 3 per cent solution of levulinic acid in water overnight as compared with soaking in water alone decreased germination from 86.6 to 10 manufacturers' directions resulted in no significant increase in growth with either radish or oat (tables 3, 7).

FIELD EXPERIMENTS

Nine different treatments of seeds with growth substance, including various commercial preparations, when tested with nine varied crops failed to produce any increased growth (table 8). These

treatments of the seeds included soaking in water solutions of growth substances, dusting with the substances in powdered talc, and application of commercial preparations of the substances.

Levulinic acid, recently suggested as a growth-regulating substance (10), was applied in a variety of ways to both seeds and plants (tables 4, 7, 9). In no instance did a statistically significant increase in growth or yield occur.

TABLE 7

TOP GROWTH OF OAT PLANTS FROM SEEDS TREATED IN GREENHOUSE WITH PREPARATIONS OF COMMERCIAL GROWTH-REGULATING SUBSTANCES (EXPERIMENT 2, FEBRUARY–MARCH) OR WITH LEVULINIC ACID (EXPERIMENT 9, APRIL–MAY)

EXP. NO.	SEED TREATMENT	GERMINATION (%)	AVERAGE DRY WEIGHT OF TOPS* (GM.)
1.	Untreated	88.8	0.079
2.	Commercial preparation A	89.3	.091
3.	Commercial preparation B	87.0	.080
4.	Commercial preparation C	91.0	.078
5.	Commercial preparation D	87.0	.082
6.	Commercial preparation E	88.8	.082
1.	Untreated	83.8	.166
2.	Soaked 12 hours in water only	86.6	.174
3.	Soaked 12 hours in 0.05% solution of levulinic acid	84.4	.164
4.	Same as 3, but 1% solution	73.8	.177
5.	Same as 3, but 3% solution	10.0	.180
6.	Same as 2, but sprayed weekly with 1 p.p.m. levulinic acid in water solution	85.0	.171
7.	Same as 3, but sprayed weekly with 1 p.p.m. levulinic acid	85.0	.168
8.	Same as 4, but sprayed weekly with 1 p.p.m. levulinic acid	78.3	.179
9.	Same as 5, but sprayed weekly with 1 p.p.m. levulinic acid	7.7	0.163

* Average dry weight = $\frac{\text{Total dry weight}}{\text{Germination percentage} \times 168}$.

U.S. Hybrid 113 and Yellow Dent corn were grown on both fertilized and unfertilized plots. Although the experiment was not designed to compare growth differences between the two, it was observed that the number of full ears of the hybrid was nearly twice the number of the dent corn. The yield of the fertilized corn was greater than that of the unfertilized. There were no significant increases as shown by analysis of variances in fresh or dry weights of stalks, number of ears, or amount of grain as a result of any of the seed treatments (table 10). Again it must be repeated that —as with the other crops—the seed treatments of the two corn varieties resulted in no statistically significant increases in growth or yield, either in the fertilized or the unfertilized crops.

Summary

1. Greenhouse experiments with radish at the U.S. Bureau of Plant Industry Station, Beltsville, Maryland, and with oat at the University of Chicago failed to indicate statistically significant increased growth of plants from seeds treated with growth-regulating substances over those not treated. Indoleacetic acid, indolebutyric acid, phenylacetic acid, naphthaleneacetic acid, and naphthalene acetamide were applied to seeds in talc dust at concentrations from 2 to 33,000 p.p.m. They were also applied by soaking the seeds 14 hours in water solutions of these substances at concentrations from 10 to 500 p.p.m. Application of levulinic acid in a variety of ways to seeds and plants failed to increase their growth over untreated ones. Commercial preparations of growth substances for seed treatment, used according to manufacturers' directions, were similarly ineffective. With radish these chemicals and mixtures were applied to seeds grown on three soil types but caused no significant increased growth of the plants on any of the soils.

2. Field experiments at Beltsville with radish, oat, turnip, sugar beet, soybean, squash, carrot, buckwheat, and corn likewise failed to indicate statistically significant increased growth of plants from seeds treated with seven growth substances, including several commercial

TABLE 8

FIELD CROP TESTS OF SEED TREATMENTS WITH GROWTH-REGULATING SUBSTANCES
GROWTH GIVEN AS AVERAGE FRESH WEIGHT OF ENTIRE PLANT

Exp. no.	Seed treatment	Carrot (gm.)	Radish (gm.)	Sugar beet (gm.)	Turnip (gm.)	Sugar beet* (Wisconsin) (kg.)	Radish (Wisconsin) (gm.)	Squash* (kg.)	Soybean* (kg.)	Buckwheat* (kg.)	Oat* (tops only) (kg.)
1.	Untreated	126.1	8.21	957.0	232.2	19.9	32.3	4.58	19.68	19.59	4.61
2.	Talc dust only	130.1	9.43	.....	.....	.....	.....	.....	.....	.....	.....
3.	Commercial dust preparation A	126.1	8.34	852.7	202.3	20.2	31.7	4.99	20.50	20.14	.....
4.	Commercial dust preparation B	133.3	7.48	857.3	227.7	.....	27.8	4.83	20.09	20.50	4.67
5.	Commercial dust preparation C	135.1	9.11	938.9	202.7	.....	32.2	4.20	21.14	19.37	.....
6.	1% naphthaleneacetic acid in dust	135.1	.....	.....	233.6	.....	.....	.....	.....	.....	.....
7.	Soaked in water 4 hours	121.5	7.57	.....	.....	.....	.....	.....	.....	.....	.....
8.	Soaked 4 hours in 0.1% naphthaleneacetic acid solution	137.8	0.00	.....	.....	.....	.....	.....	.....	.....	.....
9.	Soaked in water 14 hours	132.9	8.61	.....	.....	.....	.....	.....	.....	.....	4.40
10.	Soaked in 0.01% indolebutyric acid solution 14 hours	127.9	7.07	.....	.....	.....	.....	.....	.....	.....	4.36
11.	Soaked in 0.1% naphthaleneacetic acid solution 14 hours	.....	.....	.....	.....	.....	.....	.....	.....	.....	4.16

* Weight per plot instead of per plant.

TABLE 9

SEED TREATMENT WITH LEVULINIC ACID ON FIELD CROPS. GROWTH GIVEN AS AVERAGE FRESH WEIGHT OF ENTIRE PLANT

Exp. no.	Seed treatment	Turnip (gm.)	Sugar beet (gm.)	Carrot (gm.)	Radish (gm.)	Radish (Wisconsin) (gm.)	Squash (kg.)	Sugar beet* (Wisconsin) (kg.)	Soybean* (kg.)	Buckwheat* (kg.)	Oat* (tops only) (kg.)
1.	Untreated	232.2	957.0	126.1	8.21	32.3	4.581	19.9	19.68	19.59	4.61
2.	Soaked 4 hours in water	198.0	902.0	121.5	7.57	.....	.....	.....	21.64	.....	.....
3.	Soaked 4 hours in 0.1% levulinic acid	222.7	870.0	117.9	6.16	31.3	.....	.....	21.00	.....	.....
4.	Soaked 4 hours in 1% levulinic acid	.....	834.0	.....	.....	.....	4.65	.....	.....	.....	.....
5.	Soaked 14 hours in water	.....	.....	.....	.....	.....	.....	.....	.....	.....	4.40
6.	Soaked 14 hours in 0.1% levulinic acid	.....	.....	.....	.....	.....	.....	20.2	.....	19.19	.....
7.	Soaked 14 hours in 1% levulinic acid	.....	.....	.....	.....	.....	.....	.....	.....	.....	4.72
8.	Dusted with levulinic acid in talc, 1,000 p.p.m.	219.5	848.2	.....	.....	.....	.....	18.6	19.10	.....	.....
9.	Dusted with levulinic acid in talc, 10,000 p.p.m	229.6	961.6	.....	.....	32.65	3.647	.....	21.00	19.19	.....
10.	Dusted with levulinic acid in talc, 1000,000 p.p.m.	229.5	.....	.....	.....	.....	.....	.....	19.27	.....	.....

* Weight per plot instead of per plant.

preparations. Field experiments on sugar beet and radish were duplicated at Lake Geneva, Wisconsin, on a different soil and with different climatic conditions but with similar results.

talc; by dusting with 1 per cent levulinic acid in talc; by soaking 14 hours in 1 per cent levulinic acid; and with three different commercial preparations. The seeds were grown on both fertilized and unfer-

TABLE 10

EFFECT OF SEED TREATMENT WITH GROWTH-REGULATING SUBSTANCES ON U.S. HYBRID 113 AND YELLOW DENT CORN GROWN ON FERTILIZED AND UNFERTILIZED SOIL
FIGURES GIVEN AS AVERAGES

Variety	Soil	Seed treatment*	Fodder			Grain			
			Fresh weight per plot (kg.)	Dry weight per plot (kg.)	No. ears per plot	No. nubbins per plot (gm.)	Fresh weight per ear (gm.)	Dry weight of grain per ear (gm.)	Dry weight of cob per ear (gm.)
U.S. Hybrid	Unfertilized	1. Untreated	25.90	6.53	50.4	3.4	299	161.5	43.4
		2. Commercial preparation A	27.53	6.67	47.5	6.4	317	144.7	38.3
		3. Commercial preparation B	25.63	6.21	45.2	6.4	298	155.8	44.1
		4. Commercial preparation C	26.53	6.71	45.2	5.2	319	165.3	42.5
		5. Dusted 1% naphthaleneacetic acid	28.12	5.90	46.7	6.5	294	162.0	45.0
		6. Dusted 1% levulinic acid	25.49	5.81	49.0	3.0	310	146.0	37.4
		7. Soaked 14 hours in 1% levulinic acid	25.58	6.26	41.2	8.0	294	156.6	43.5
	Fertilized	1. Untreated	30.25	6.08	45.1	7.4	342	191.1	47.7
		2. Commercial preparation A	30.39	5.62	47.6	6.0	340	185.8	45.2
		3. Commercial preparation B	29.66	5.94	48.8	6.1	341	185.4	46.5
		4. Commercial preparation C	30.75	6.35	51.6	5.8	339	185.2	46.9
		5. Dusted 1% naphthaleneacetic acid	29.53	6.12	47.8	4.4	331	187.7	44.9
		6. Dusted 1% levulinic acid	31.16	6.17	50.0	5.3	349	196.0	46.9
		7. Soaked 14 hours in 1% levulinic acid	30.48	5.99	49.3	6.6	346	202.7	41.9
Yellow Dent	Unfertilized	1. Untreated	26.81	7.17	23.0	9.5	322	142.6	41.6
		2. Commercial preparation A	29.53	7.35	24.5	9.8	317	152.8	46.9
		3. Commercial preparation B	30.57	7.94	24.8	9.2	345	135.6	33.2
		4. Commercial preparation C	30.89	7.89	23.0	7.0	351	138.4	42.1
		5. Dusted 1% naphthaleneacetic acid	27.76	7.53	22.4	6.2	305	144.0	39.9
		6. Dusted 1% levulinic acid	29.12	7.39	22.0	10.0	311	128.5	39.3
		7. Soaked 14 hours in 1% levulinic acid	26.04	7.32	17.2	8.2	366	151.6	46.4
	Fertilized	1. Untreated	31.75	7.98	27.5	9.8	308	162.2	45.0
		2. Commercial preparation A	30.71	7.26	23.5	7.0	360	163.8	43.9
		3. Commercial preparation B	31.52	7.08	23.7	7.0	323	169.3	43.4
		4. Commercial preparation C	31.34	7.30	23.8	10.7	345	162.2	43.6
		5. Dusted 1% naphthaleneacetic acid	33.79	8.75	24.7	11.4	344	173.4	51.5
		6. Dusted 1% levulinic acid	34.29	8.25	24.8	7.1	342	171.8	43.5
		7. Soaked 14 hours in 1% levulinic acid	33.11	8.48	28.0	9.4	339	170.3	44.7

* Analyses of variance were made on treatments which showed an increase over the untreated. None of the differences was significant at the 5% level.

3. Levulinic-acid treatments applied in a number of ways to seeds of these crops likewise failed to increase their growth.

4. Seeds of U.S. Hybrid 113 and Yellow Dent corn were treated by dusting with 1 per cent naphthaleneacetic acid in talc; by dusting with 1 per cent levulinic acid in talc; by soaking 14 hours in 1 per cent levulinic acid; and with three different commercial preparations. The seeds were grown on both fertilized and unfertilized soil. Measurement of stalk and grain yield failed to indicate any significant increase as a result of any of the treatments.

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CHEMICAL CHANGES ACCOMPANYING GROWTH AND DEVELOPMENT OF SEED AND FRUIT OF THE ELBERTA PEACH[1]

F. A. LEE AND H. B. TUKEY

(WITH ONE FIGURE)

Introduction

The peach fruit develops in three stages (1, 2). In stage I the pericarp of the Elberta variety increases in size for a period of about 49 days following full bloom. During stage II it makes only retarded development, at the same time that the embryo reaches nearly maximum size. During stage III, known as the "final swell," the fleshy pericarp increases rapidly in size to fruit ripening.

The present study was undertaken to determine the gross chemical changes that take place under natural growing conditions during this development, both as regards the fleshy and the stony portions of the pericarp, and also the integuments, nucellus, endosperm, and embryo, in so far as possible. This paper is a supplement to a previous one (2) in which chemical changes taking place under artificial conditions of ringing and defoliation were studied. In both papers, special emphasis has been placed on the development of the embryo in relation to the development of the fruit.

Experimental procedure

Studies were conducted during the growing seasons of 1935, 1936, and 1937, in the varietal orchards of the New York State Agricultural Experiment Station and in the commercial orchard of Willis Henderson at Dresden, New York. These were good crop years, providing excellent growing conditions for the samples collected.

Measurements of development of the embryo, endosperm, nucellus, integuments, stony pericarp, and fleshy pericarp were recorded at fortnightly intervals, from which the correlations with the chemical analyses were made.

Chemical analyses were made of fleshy pericarp, stony pericarp, integuments, nucellus, endosperm, and embryo, in so far as possible. Early in stage I the embryo is microscopic, and quantities sufficient for chemical analysis could not be secured. During the early part of stage I also it was not possible to make a clean separation of fleshy from stony pericarp, and so for one sampling these two tissues were taken together. Again, during the early part of stage II it was not practicable to separate nucellus and endosperm, and so these tissues were taken together for one sampling. Partially to compensate for this situation, analyses were made of the entire seed during stage I and early stage II, offering the possibility of arriving at values for some of these tissues by subtraction.

The samples were weighed immediately after their separation and divided into four duplicate lots for determinations. Speed in the preparation of samples was found essential to prevent loss of moisture and also to reduce possible chemical changes. It was necessary to employ

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TABLE 1

CHEMICAL ANALYSES OF TISSUES OF FRUIT AND SEED OF ELBERTA PEACH FROM EARLY STAGES TO FRUIT MATURITY (PERCENTAGE FRESH-WEIGHT BASIS)

Stage of fruit development	Date	Percentage					
		Moisture	Reducing substances calculated as glucose	Sucrose	Ether extract	Nitrogenous substances (N×6.25)	Ash
		Stony and fleshy pericarp*					
I	June 11	85.68	4.02	0.50	0.91	2.15	.....
		Fleshy pericarp					
I	June 22	86.30	4.33	0.46	0.68	1.56	0.41
II	July 8	84.92	4.26	1.35	0.39	1.39	0.38
II	July 21	84.80	3.81	1.43	0.65	1.33	0.56
II	Aug. 3	85.85	3.64	1.83	0.52	1.75	0.54
III	Aug. 19	86.32	4.24	1.86	0.26	1.14	0.61
III	Sep. 11	88.46	3.53	3.37	0.14	0.82	0.32
III	Sep. 27	87.05	2.94	6.93	0.15	0.83	0.34
		Stony pericarp					
I	June 11	.....	.....	.....	.....	.....	.....
I	June 22	89.74	4.71	0.21	0.55	1.52	0.31
II	July 8	84.71	3.74	0.58	0.52	1.71	0.45
II	July 21	56.52	1.60	0.38	0.33	2.04	0.44
II	Aug. 3	42.35	0.77	0.25	0.22	2.26	0.32
III	Aug. 19	30.04	0.64	0.11	0.18	1.15	0.21
III	Sep. 11	24.71	0.35	0.12	0.11	0.67	0.15
III	Sep. 27	23.47	0.39	0.13	0.13	0.65	0.13
		Entire seed					
I	June 11	91.73	0.94	0.37	0.75	3.13	.....
I	June 22	93.20	0.93	0.28	0.51	2.28	0.66
II	July 8	93.05	0.49	0.17	0.58	2.56	0.56
		Integument					
I	June 22	88.07	1.33	0.44	0.70	4.11	.....
II	July 8	87.75	0.55	0.20	0.67	4.96	0.97
II	July 21	83.18	0.89	0.33	0.54	4.11	1.03
II	Aug. 3	76.00	1.39	1.33	1.70	5.03	1.26
III	Aug. 19	73.15	1.78	1.94	3.17	4.43	1.10
III	Sep. 11	65.20	1.64	0.27	6.42	4.44	1.03
III	Sep. 27	47.45	2.06	0.57	9.64	7.04	1.34

* Tissues not sufficiently defined to separate in this one sample.

TABLE 1—*Continued*

Stage of fruit development	Date	Percentage					
		Moisture	Reducing substances calculated as glucose	Sucrose	Ether extract	Nitrogenous substances (N×6.25)	Ash
		Nucellus and endosperm					
II	July 21	94.86	0.47	0.13	0.25	2.97	0.41
		Embryo					
II	July 21	92.62	0.57	1.04	0.34	3.98	0.57
II	Aug. 3	86.45	0.35	0.50	2.52	8.27	0.76
III	Aug. 19	66.40	0.75	1.06	11.70	13.95	1.53
III	Sep. 11	42.47	0.71	1.40	29.14	16.65	1.94
III	Sep. 27	40.68	0.37	2.18	29.40	19.47	1.92

five to ten persons, working rapidly, particularly for the preparation of the samples of the smaller tissues, as integuments.

Determinations were made for moisture, reducing substances as glucose, sucrose, ether extract (fat), total nitrogen, and ash (mineral matter). The reducing substances calculated as glucose were further treated with Fleischmann's starch-free yeast to determine the proportion fermentable with yeast.

The samples for fat were dried at 95° C. for 2 hours and then placed in a vacuum at 70° until they reached constant weight. The percentage of moisture was determined from these samples. The samples for sugar were placed directly into 80 per cent alcohol and boiled slowly for 15 minutes before setting away. During the analyses for sugars these substances were extracted by means of 80 per cent alcohol, in the usual way (4). The acid in the samples of fleshy pericarp was neutralized with calcium carbonate before heating. The Munson-Walker method by direct weighing was used for all sugars. Sucrose was inverted by means of invertase, as was done in the analyses presented previously (2, 3). For nitrogen, the Gunning method was used (4), and fat was determined by the usual Soxhlet extraction method (4).

Calculations were made both on the unit-weight basis and in percentage based upon fresh weight.

Results

Although analyses were made of material for the seasons of 1935, 1936, and 1937, only those for 1937 are presented here, inasmuch as the data from the 1935 and 1936 series closely approximate those for 1937, and the latter are more complete. Mineral matter, however, was determined only for the 1937 series. The data are given on the percentage fresh-weight basis in table 1 and on the unit fresh-weight basis in table 2.

MOISTURE.—The moisture content of the fleshy pericarp on the unit basis was found to follow the general curve for

TABLE 2

CHEMICAL ANALYSES OF TISSUES OF FRUIT AND SEED OF ELBERTA PEACH FROM EARLY STAGES TO FRUIT MATURITY (UNIT-WEIGHT BASIS [MG.] OF FRESH MATERIAL)

Stage of fruit development	Date	Milligrams						
		Unit weight	Moisture	Reducing substances calculated as glucose	Sucrose	Ether extract	Nitrogenous substances (N×6.25)	Ash
		Fleshy and stony pericarp						
I.....	June 11	2499.7	2149.5	100.4	12.5	24.7	53.6	.....
		Fleshy pericarp*						
I.....	June 22	5757.0	4968.0	259.3	26.5	39.7	89.9	28.6
II.....	July 8	13615.7	11555.0	580.0	183.8	55.3	189.1	52.0
II.....	July 21	15810.3	13410.0	606.0	227.4	102.8	210.7	88.6
II.....	Aug. 3	16746.5	14385.0	610.0	306.3	87.1	292.9	90.4
III.....	Aug. 19	29435.0	25430.0	1248.3	547.2	76.6	335.8	179.6
III.....	Sep. 11	64573.4	57200.0	2272.0	2175.0	94.0	532.1	206.8
III.....	Sep. 27	75645.0	65875.0	2408.0	5059.0	113.6	628.1	257.3
		Stony pericarp						
I.....	June 11	.....	.....	.....	.....	.....	.....	.....
I.....	June 22	2760.0	2477.0	130.0	5.8	15.7	42.0	7.1
II.....	July 8	4708.3	3995.0	176.1	27.3	25.9	80.2	21.2
II.....	July 21	5135.0	2901.0	82.2	19.5	16.9	104.6	22.6
II.....	Aug. 3	5087.4	2157.0	39.3	12.7	11.2	115.1	16.3
III.....	Aug. 19	5432.5	1634.0	34.8	6.0	9.8	62.5	11.4
III.....	Sep. 11	5990.3	1344.0	21.0	7.2	6.0	40.3	9.0
III.....	Sep. 27	5237.5	1229.0	20.4	6.8	6.8	34.6	6.8
		Entire seed						
I.....	June 11	83.9	76.9	0.8	0.3	0.6	2.6	.....
I.....	June 22	380.7	355.4	3.5	1.7	1.9	8.7	2.5
II.....	July 8	647.4	602.0	3.2	1.1	3.0	16.6	3.6
		Integument						
I.....	June 22	97.0	85.6	1.3	0.4	0.7	4.0	.....
II.....	July 8	196.5	170.4	1.1	0.4	1.2	9.8	1.9
II.....	July 21	229.0	190.5	2.0	0.8	1.2	9.4	2.4
II.....	Aug. 3	151.6	115.3	2.1	2.0	2.6	7.6	1.9
III.....	Aug. 19	124.5	91.2	2.2	2.4	4.0	5.5	1.4
III.....	Sep. 11	86.8	81.2	1.4	0.2	8.0	3.9	0.9
III.....	Sep. 27	51.1	24.3	1.1	0.3	4.9	3.6	0.7

* Tissues not sufficiently defined to separate in this one sample.

TABLE 2—*Continued*

Stage of fruit development	Date	Milligrams						
		Unit weight	Moisture	Reducing substances calculated as glucose	Sucrose	Ether extract	Nitrogenous substances (N×6.25)	Ash
		Nucellus and endosperm						
II.....	July 21	177.7	168.6	0.8	0.2	0.4	5.1	0.8
		Embryo						
II.....	July 21	214.1	198.4	1.2	2.3	0.7	8.5	1.2
II.....	Aug. 3	511.4	442.3	1.8	2.6	12.9	42.3	3.9
III.....	Aug. 19	537.8	357.2	4.0	5.7	62.9	74.9	8.6
III.....	Sep. 11	565.3	228.1	4.0	7.9	156.6	95.3	11.0
III.....	Sep. 27	574.8	234.1	2.1	12.5	169.2	111.9	11.1

growth of the fruit. During stage I it increased more than five times; during stage II it increased only slightly; and during stage III it increased markedly to more than five times that at the beginning of stage III. The actual percentage of moisture remained at a fairly constant level throughout all stages, however, with some increase toward the end of stage III.

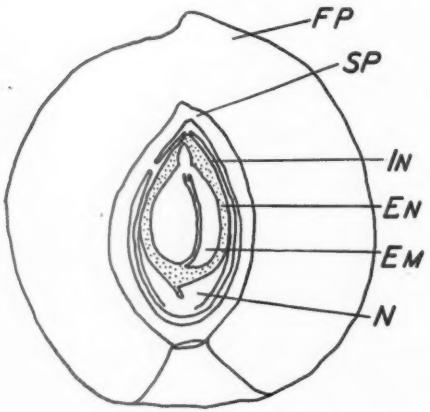

FIG. 1.—Longitudinal section of developing peach showing parts included in chemical analyses. *fp*, fleshy pericarp; *sp*, stony pericarp; *in*, integuments; *n*, nucellus; *en*, endosperm; and *em*, embryo.

Moisture in the stony pericarp decreased progressively with advancing age of the material on the percentage basis, but on the unit basis there was an increase in moisture during stage I accompanying increase in size, followed by a steady decrease during stages II and III, accompanying differentiation and hardening. The significance of these results is treated more fully in a previous paper (2), in which the role the stony pericarp may play in embryo development is discussed.

On the percentage basis the moisture content of the integuments became progressively lower, especially during stage III, while on the unit basis it showed increases up to the middle of stage II, followed by gradual decreases thereafter, accompanying drying out and maturing of the tissues. The percentage moisture in the embryo showed a steady decline,

from the time in stage II when samples could be secured until the end of stage III; while on the unit basis there was marked increase in moisture during stage II, followed by decline during stage III accompanying the differentiation of tissues and the accumulation of storage materials.

ETHER EXTRACT (FAT).—The amount of ether extract (fat) by both systems of calculation was irregular for the fleshy pericarp. On the percentage basis it declined during stage I, increased by a small degree during stage II, and decreased further to a constant level by the middle of stage III. On the unit basis it increased during stage I and the first part of stage II, then declined until the middle of stage III, when it again increased until fruit ripening. The stony pericarp showed progressive decrease in ether extract on the basis of percentage throughout all three stages. On the unit basis it showed an increase during stage I, followed by a progressive decrease during stages II and III. For the integuments, on the percentage basis it declined slightly but consistently until toward the end of stage II, when it increased steadily and appreciably to fruit ripening. On the unit basis it increased slowly during stages I and II, increased rapidly during the first part of stage III, and then declined toward the end of that stage. The embryo, which contains a relatively high percentage of ether extract at maturity, showed increase during all three stages, both on the percentage basis and the unit basis.

REDUCING SUBSTANCES CALCULATED AS GLUCOSE.—Reducing substances calculated as glucose require special mention because aliquots of each sample were treated with starch-free yeast and reducing substances again determined. In all instances all except traces of these materials were fermented, indicating that these substances are definitely reducing sugars.

On the unit-weight basis reducing substances accumulated steadily in the fleshy pericarp during stage I, slightly during stage II, and markedly during stage III. On the percentage basis they were irregular, remaining fairly constant during stage I, declining slightly during stage II, increasing during the first part of stage III, and then declining as the end of that stage approached. With the stony pericarp, on the unit basis they were relatively high during stage I, increasing still further during the period, but declining steadily during stages II and III at a time when this tissue was differentiating and hardening. On the percentage basis the decline was steady during all three stages to fruit ripening. With the integuments, the results were variable for both methods of expression. On the percentage basis they showed a decline from stage I to stage II, an increase during stage II, a decline during the first part of stage II, followed by an increase toward the end of stage III. On the unit basis there was a slight decline from stage I to stage II, a slight increase during stage II, and a decline during stage III. For the embryo, on the percentage basis they declined while the embryo was making rapid increase during stage II, then increased as storage materials accumulated at the beginning of stage III; and declined toward the end of stage III. On the unit basis they increased slightly during stage II, sharply during the first part of stage III, and then dropped sharply as fruit ripening approached.

SUCROSE.—Sucrose increased steadily though slowly during stages I and II, as calculated on the unit basis, and increased greatly during stage III to more than fifteen times what it was at the be-

ginning of that stage. When calculated on the percentage basis the sucrose content increased slowly at first, followed by rapid increase. In the stony pericarp the trend is similar for both methods of expression, namely, increase during stage I followed by steady decline during stage II to a low level, which was maintained at that point throughout stage III. The integuments showed a somewhat similar trend to that for the stony pericarp by both methods of calculation. For the embryo, there was a slight increase in sucrose on the unit basis during stage II, and marked and rapid increase during stage III. On the percentage basis there was slight decline during stage II and increase during stage III.

NITROGENOUS SUBSTANCES.—Nitrogenous substances ($N \times 6.25$) declined rather steadily for the fleshy pericarp during all three stages on the percentage basis, while on the unit basis the reverse was true. The increase in nitrogenous substances was particularly rapid and great in amount during stage III. For the stony pericarp, on both the unit and percentage basis there was an increase during stage I, slight increase during stage II, and a sharp and steady decline during stage III to fruit ripening. The integuments showed an increase on the unit basis during stage I, followed by a decline during the last of stage II and throughout stage III as the tissue differentiated. On the percentage basis it remained at a fairly constant level until the end of stage III, when it increased as the tissues lost moisture. The embryo showed substantial increases by both methods of expression throughout all three stages.

STARCH.—Qualitative tests, as reported previously (2), showed starch negative in the embryo, stony pericarp, and ripe fleshy pericarp, although small amounts were found in the fleshy pericarp in early stages of fruit development when the fruit was green.

ASH (MINERALS).—The mineral constituents, as represented by ash content, increased steadily in the fleshy pericarp on the unit basis. On the percentage basis they increased slightly until the beginning of stage III and then sharply declined. In the stony pericarp, on both the unit and percentage basis the amount increased during stage I, began to decline toward the end of stage II, and continued steady decline until fruit ripening. In the integuments the same relation prevailed on the unit basis, but on the percentage basis the ash increased during stage II, declined slightly during the first part of stage III, and increased as the end of the stage was reached. In the embryo, by both methods of calculation the ash content increased steadily during stage II and the first part of stage III, and remained at that point until fruit ripening.

Discussion

The general pattern for chemical composition of fruit and seed parts of the peach during development is similar to that for much plant material. Especially is this true when the data are correlated with morphological development.

For example, during the first part of stage I, when active growth is taking place in the fleshy and stony pericarp and nucellus and integuments, and at a time when some of this growth is by cell division, the percentage moisture is relatively high and such labile forms as reducing sugars are also fairly high in amount. During stage II, when little increase in size occurs in the fleshy portion of the pericarp, changes in composition are small. It is during this period, however, that thickening and hardening of the cell walls of the stony portion of the pericarp

begin, so that there is steady decline in percentage moisture, reducing sugars, sucrose, ether extract, and ash in that portion.

Further, active growth of the embryo begins during this stage, correlated with high moisture level and low content of such storage forms as ether extract and nitrogenous substances. During stage III, when increase in size of the fleshy pericarp is rapid, caused largely by cell expansion, the sucrose content increases markedly and the moisture content slightly; while the percentages of reducing substances, ether extract, nitrogenous substances, and ash decline. On the other hand, since the cell walls of the stony pericarp thicken and harden still further during this period, and since it is not an organ for the storage of such materials as were included in the analyses, the percentages of moisture, reducing substances, sucrose, ether extract, nitrogenous substances, and ash decline to the lowest level observed during development. At the same time, in the embryo, which at fruit ripening contains a high percentage of such storage forms as ether extract and nitrogenous substances, rapid accumulation of these materials continues while the moisture declines.

The data show especially well in this way the necessity for careful separation of plant tissues and parts intended for chemical analyses, rather than the combining of related parts, as has so frequently been done. A final example is shown in the development of the seed. During stage I it consists almost entirely of nucellus and integument, which have high moisture content. As development proceeds the integuments lose moisture, the nucellar tissue is digested by the endosperm, and the endosperm is in turn digested and replaced by the embryo—which finally accumulates storage materials in quantity. Accordingly, analyses of the total seed at different periods in development are really analyses of widely different tissues.

Summary

1. The moisture content, reducing substances calculated as glucose, sucrose, ether extract (fat), total nitrogen, and ash (minerals), are given for the fleshy pericarp, stony pericarp, integument, nucellus and endosperm, and embryo at regular intervals during the growing season at such times as the parts analyzed were of sufficient size to permit adequate sampling.

2. The analyses are correlated with morphological development of the plant parts analyzed.

3. The data show the importance of careful separation of tissues intended for analyses and the value of considering the morphological nature of developing plant parts in connection with such analyses.

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HISTOLOGICAL STUDY OF FREEZING, DESICCATION, AND WINTER INJURY OF RASPBERRY CANES[1]

AUSTIN O. SIMONDS

(WITH EIGHT FIGURES)

Introduction

Winter injury to many varieties of raspberries is of common occurrence in northern Colorado, and is attributed to a number of causes. Prominent among the causes are the breaking of certain tissues subsequent to the formation of ice crystals, protoplasmic death resulting directly from low temperatures, and injury of the protoplasm owing to extreme desiccation. In this latter regard, BRIERLEY[2] reports that "it is probable that many cases of winter injury reported for otherwise hardy raspberries have been due to desiccation." He found that winter injury occurred when the moisture content of the plant fell below 50 per cent of the dry weight and considered that this condition was brought about when deep freezing of the soil interfered with normal conduction in the canes.

A study was made of normal and injured raspberry canes in order to determine the types of tissue injuries resulting from apparent winter injury in the field, and to compare these with artificially induced injuries resulting from both freezing temperatures and slow desiccation at temperatures above freezing.

[1] Published with the approval of the Director as Paper no. 154, Scientific Journal Series, Colorado Agricultural Experiment Station.

[2] BRIERLEY, W. B., Desiccation in the Latham raspberry. Amer. Soc. Hort. Sci. Proc. 31:Supl. pp. 110–113. 1934.

Material and methods

FIELD STUDIES

Apparently uninjured Latham raspberry canes were obtained at random from field plantings of the Horticulture Section, Colorado Agricultural Experiment Station, on March 17, 1938. A second collection was made from the same field on June 27, after evidence of winter injury was apparent. Samples from these collections were cut into small pieces with a fine-toothed electric rotary saw, prior to placing them in the fixing agent. This method prevented crushing of the outer stem tissues. The teeth of the saw produced a shallow roughened end, which was discarded at the time of sectioning.

The material was fixed in formalin-acetic alcohol (7 cc. formalin, 5 cc. glacial acetic acid, and 88 cc. of 50 per cent ethyl alcohol). A preliminary treatment of 1–15 minutes in a solution containing 0.5–1 per cent sodium hypochlorite was found desirable to remove the brown tannin-like substance present in the phloem and inner cortical cells of mature canes. The required concentration of chlorine in the solution and the length of time necessary to clear the cells of this color varied, apparently depending upon the age of the section and its proximity to the tip of the cane. For purposes of comparison, however, some samples were prepared without use of the hypochlorite solution. Following decolorizing, sam-

ples were treated with hydrofluoric acid, imbedded in celloidin, and cut on a sliding microtome. Other pieces were imbedded without the hydrofluoric-acid treatment (which was found unnecessary), while still others were cut on the microtome without either imbedding or treating with the acid. The best preparations were most consistently obtained from material imbedded in celloidin. Of the several staining combinations used, fast green F.C.F. with safranin O proved most satisfactory and was used most frequently.

Artificially induced injuries

To determine the results of artificially induced freezing and desiccation, six plants averaging four canes each were transplanted to soil in large cans on November 4, 1939. Two of these plants were then subjected to continuous refrigeration at 8° F. for 12 weeks. Two other plants were placed in the greenhouse and allowed to approach air dryness at temperatures above freezing. The remaining two plants were alternated between the refrigerator (8° F.) and the greenhouse (75° F.) at 7-day intervals for the first 8 weeks and at 14-day intervals for the remaining 4 weeks. Water was added only to the plants given the alternating treatment.

Samples were taken at the end of 4 weeks from plants left to desiccate, and at the end of 2, 3, 5, 10, and 12 weeks from those in continuous refrigeration. Samples were taken from plants given the weekly alternating treatment as follows: 1-week refrigeration and 1-week greenhouse; 2-weeks refrigeration and 1-week greenhouse; 3-weeks refrigeration and 2-weeks greenhouse; 5-weeks refrigeration and 5-weeks greenhouse; and 6-weeks refrigeration and 6-weeks greenhouse. The same methods of preparation were used as described for field material.

Further studies of the effect of freezing were conducted by taking 6-inch pieces of green plants from the field on July 24, August 1, and September 19, 1940, and freezing them in the refrigerator at 23° F. for 1 week before making sections. Samples taken from the middle of these pieces were immediately cut into sections without imbedding. These sections were fixed in 50 per cent ethyl alcohol for 2 hours before being treated with the sodium-hypochlorite solution and before being stained. Other pieces were placed in the formalin-acetic-alcohol fixing agent and sectioned the following day.

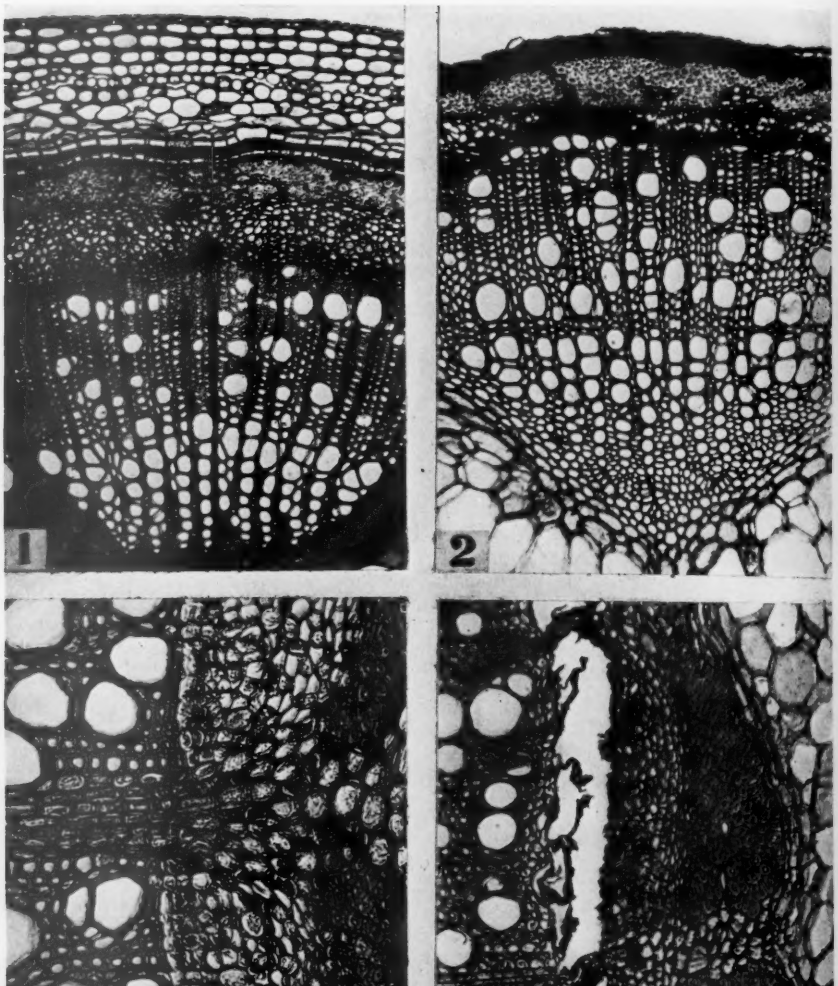
Observations

Naturally occurring field injuries

Many samples collected in 1938 showed no abnormal conditions in the tissues of the stem. However, some sections of canes did show a distinct breaking of cell walls in the outer tissues. These breaks were of a nature that precluded the possibility of mechanical injuries in the process of cutting the sections.

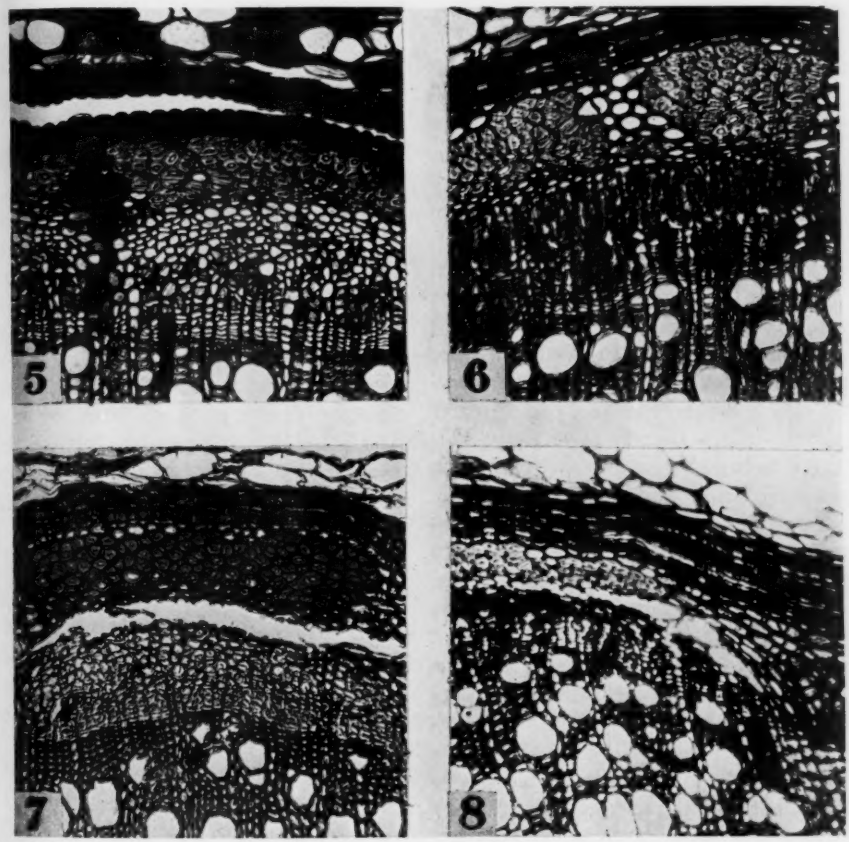
One type of injury was the presence of broken cambial cells. The rupture occurred in the radial walls of the cells, so that broken areas extended tangentially and parallel to the outermost cells of the secondary xylem (fig. 3). A second type of break was evident in the outer region of the primary phloem adjacent to the pericyclic fibers. The ruptured area in general extended tangentially (fig. 7). Frequently radial breaks were found to extend down to the side of the multiseriate rays. More rarely the tissue of the phloem was split down one of the uniseriate rays near the center of a large bundle.

The outer portions of the cortex and epidermis were frequently found to be



FIGS. 1–4.—Fig. 1, normal cane from field (Oct. 4, 1940). Fig. 2, section from dead cane, field collection, showing tannin-like substance in blackened tissues. Fig. 3, broken cambial layer found in field sample. Fig. 4, broken cells of cambial region produced artificially by freezing green canes.

broken off in flakes. Occasionally such breaks appeared in the small, thin-walled parenchymatous cells just outside the abscission layer.[3] More commonly the separation was found in the thin-walled cells of the abscission layer, formed between two layers of thick-walled lignified cells. This caused the outer tissues to be shed in large flakes (fig. 5). The latter condition was not considered an injury but the normal abscission of the plant tissue. The abscission layer was formed in the cortex just outside the pericycle (fig. 1).



FIGS. 5–8.—Fig, 5, section from field sample (Feb. 2, 1940) showing normal phloem condition with splitting through abscission layer. Fig. 6, necrosis of outer phloem from dormant plants alternated weekly between refrigerator and greenhouse. Fig. 7, broken area in primary phloem from field sample. Fig. 8, artificially produced break in primary phloem in canes refrigerated for 10 weeks continuously.

Rupture of the tissue was not evident in some of the field material, but phloem necrosis was evident as a noticeable breakdown of walls of many sieve tubes. These walls were indistinct, as if partially dissolved, suggesting enzymatic action.

[3] HABERLANDT, G., Physiological plant anatomy. pp. 143–144. 1914.

This injury was usually accompanied by deposition of much brown tannin-like material in all the living cells outside the cambium. The presence of such tannin-like material was also observed in dead canes taken from the field (fig. 2).

Artificially induced injuries

Alternate freezing and thawing did not break dormancy in the transplanted plants, and the ruptured-tissue type of injury was not evident. (Evidence of the failure to break dormancy was in the lack of new cell formation by the cambium and the uniform staining reaction of cells adjacent to the cambium.) Phloem necrosis was most pronounced in the plants subjected to periods of alternate freezing and thawing. Plants exposed to twelve alternate weekly periods in the refrigerator and in the greenhouse showed greater phloem injury (fig. 6) than those subjected to two alternate weekly periods in the refrigerator and in the greenhouse. Plants given treatments intermediate between these extremes (three alternate weekly periods in the refrigerator and two in the greenhouse) showed various results. Some canes from plants given the latter treatment showed no more injury than those subjected to the shortest alternate treatment, while others revealed more injury. Frequently a vascular bundle showing normal phloem was found between the necrotic areas.

Injuries resulting from continuous freezing.—Rupture of the primary phloem cells was found in sections from plants left in continuous refrigeration for 10 and 12 weeks (fig. 8). The breaks appeared to start in cells adjacent to the multiseriate rays and to spread tangentially across the bundles to the opposite sides. Some phloem necrosis was evident but was not so noticeable as in canes given the alternate freezing and thawing treatment.

Fresh green canes with leaves attached were removed from the field in July and August, 1940, and subjected to continuous refrigeration for 1 week (fig. 4). Rupture of the cambial region was then observed. Green samples having leaves, taken from the field September 17, 1940, and subjected to the same treatment, did not show a broken cambium.

Injuries resulting from desiccation.—Plants allowed to dry out exhibited no broken cells. Although the tissues were intact, the sieve-tube contents appeared to be more shrunken than those observed in normal material. Further, in the tissues of desiccated plants the entire region outside the cambium was darker and contained more of the tannin-like material than was observed in the nondesiccated control material. Finally, the break in the abscission layer was more pronounced in the case of the desiccated canes.

Summary

1. Histological studies were made of uninjured and apparently winter-injured raspberry canes in the field. In addition, similar studies of artificially induced injuries were made after subjecting canes to continuous freezing, alternate freezing and thawing, and desiccation above freezing temperatures.

2. Field injuries believed to have been caused by freezing compared favorably with both types of artificially induced freezing injuries. Phloem necrosis, phloem rupture, cambial rupture, and the deposition of tannin-like material in the outer parenchymatous stem tissues were characteristic features of the field material. Injuries due to alternate freezing and thawing were principally char-

acterized by phloem necrosis. Canes subjected to continuous refrigeration revealed phloem rupture in dormant canes and ruptured cambial cells in nondormant ones, accompanied by limited phloem necrosis.

3. Field injuries did not show the same type of tissue impairment as found in canes subjected to desiccation above freezing temperatures. The latter type of injury was characterized by marked deposition of the tannin-like material, reduction in size and shrinkage of the contents of all living cells outside the cambium, and pronounced splitting of abscission layers.

The writer is indebted to GEORGE BEACH, Associate Horticulturist, Colorado Agricultural Experiment Station, for calling attention to this problem and furnishing the material.

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EFFECTS OF VAPOROUS NAPHTHOXYACETIC ACID ON DEVELOPMENT OF TOMATO FRUITS, WITH SPECIAL REFERENCE TO THEIR VITAMIN C CONTENT

JOHN W. MITCHELL[1] AND MURIEL R. WHITEHEAD[2]

(WITH TWO FIGURES)

The use of growth-regulating substances for the purpose of increasing fruit set has recently been suggested as a possible aid in the commercial production of greenhouse tomatoes. Several methods of applying the treatments have been used, including spraying the flowers with emulsions containing the growth substances; smearing the styles with a paste containing them; or applying the substance as a vapor to the buds and newly opened flowers (**3, 5, 7**). Seedless fruits that result from such treatments are reported to set well and to grow to a slightly larger size than those that develop from untreated flowers (**3, 5**). The color and flavor of these artificially induced parthenocarpic fruits are comparable with those of field-grown seeded ones.

Since it has been shown that seed development is greatly reduced (**2, 5, 6**) and the amount of gelatinous pulp increased in tomato fruits as a result of treating the flowers with growth-regulating substances, it was considered of interest to determine whether these anatomical changes in the fruit were associated with noticeable differences in some constituents of nutritional interest. The vitamin C and ash contents of fruits from pollinated flowers were compared with those of fruits from flowers which had been treated with vaporous β-naphthoxyacetic acid just prior to pollination.

[1] Physiologist, [2] Junior Plant Physiologist; U.S. Bureau of Plant Industry Station, Beltsville, Maryland.

Material and methods

Ninety Bonny Best tomato plants of uniform size were grown in a greenhouse in soil contained in 10-inch clay pots. The plants were arranged in rows extending across the benches, a row of treated plants alternating with a row of untreated ones. Three treated and three untreated rows constituted a block, the entire experiment being made up of five blocks distributed throughout the house. In gathering samples for analyses, equal numbers of treated and control fruits were gathered at random from each block.

The most effective time at which to apply the treatment was determined by means of preliminary experiments, which showed that the greatest number of seedless fruits (50–70 per cent) developed when the flowers were treated at the time of pollination or 1–5 days previously. Very few seedless fruits were developed when the treatment was applied 3–7 days after pollination. When the plants of the present experiment had grown until one flower on the first cluster was fully open and a second beginning to open (fig. 1), the plants to be treated were moved from the greenhouse to an adjacent room (approximately 2500 cubic feet capacity)

which could be tightly closed. They were treated by vaporizing β-naphthoxyacetic acid (250 mg./1000 cubic feet) on a hot glass plate and allowing the plants to remain in the closed, vapor-filled room overnight. The plants were then returned to their original positions on the greenhouse benches. An equal number

FIG. 1.—Cluster of tomato flowers showing stages of opening when naphthoxyacetic acid vapor was applied.

of control plants were allowed to remain in the greenhouse away from the vapor. Each flower of both treated and control plants was carefully pollinated when open and tagged with a record of its stage of development at the time of treatment. The fruits were then allowed to grow and ripen during the months of February, March, and April.

In another experiment fifty selected Marglobe tomato plants were grown in a greenhouse section with a capacity of approximately 4000 cubic feet. These plants were treated at intervals with vaporous naphthoxyacetic acid as previously described, except that all the plants were left in place and the chemical volatilized in the closed section. Treatment was applied when flowers on the first cluster of a majority of the plants had reached the stage of development shown in figure 1. Identical treatments were given flowers of the second and third clusters when they had reached this stage of development, while the fourth cluster was left untreated. In this experiment none of the flowers were hand pollinated, but some natural pollination doubtless occurred.

The soil in which the plants were grown was frequently fertilized with liquid manure, and the plants grew vigorously during both experiments.

Ripeness was designated as the stage of maturity attained on the day the fruit was completely red in color. Only ripe fruits were collected for determinations of percentage solids and the ash and vitamin C content. The vitamin determinations were made immediately after the fruits were picked by cutting a median slice, approximately 1 cm. in thickness, from each fruit (4). The method used to determine the vitamin content of these sections is described by DAVIS (1).

The percentage of solids and the ash content were determined in aliquots taken from fruits that had been finely ground in a blender. For moisture determinations the samples were dried at 80°–85° C. for approximately 18 hours in a well-ventilated oven, then the residue was ignited in a muffle furnace for ash content.

Results

GUSTAFSON (3) has shown that fruit set on field-grown plants was increased as a result of the application of naphthoxyacetic acid paste to the cut surface of the styles of tomato flowers, and HOWLETT (5) has demonstrated that fruit set in a greenhouse was improved when pollination was supplemented by the application of indolebutyric acid to the severed styles. This effect was especially noticeable when conditions were not favorable for pollination (5).

TABLE 1

RESPONSES OF TOMATO FRUITS OF FIRST CLUSTER TO TREATMENT WITH VAPOROUS NAPHTHOXYACETIC ACID. ASH AND VITAMIN CONTENT EXPRESSED AS AVERAGE OF FIFTEEN DETERMINATIONS

TREATMENT	FRUIT SET*		FRUIT NOT FULLY SEEDED (%)	SEEDLESS FRUIT (%)	AVERAGE FRESH WEIGHT OF RIPE FRUIT (GM.)	ASH (%)	VITAMIN C CONTENT (MG/100 GM. FRESH WEIGHT)
	FIRST TWO BLOSSOMS OPEN (%)	REMAINDER OF BLOSSOMS (%)					
Control..	84	33	9	6	152	0.49	17.7
Treated..	78	45	80	50	152	0.47	18.9

* Differences of 11 required for significance for odds of 19 to 1.

In the present experiment, individual flowers of a cluster responded differently to treatment with respect to the number of fruits set. Somewhat fewer fruits developed from the first two flowers that opened in the first cluster on treated plants than from the first two flowers in a corresponding cluster on untreated plants, but this difference was not statistically significant. On the other hand, treatment significantly increased the number of fruits that developed from the remaining flowers in the first clusters (table 1), although most of these were tightly closed at the time of vaporizing and did not open until several days later. Fruit set on the second clusters was not significantly affected by treatment. The effect on fruit set in the third clusters was not measured, since their flowers were even more immature at the time of treatment than were those on the second clusters.

There was a marked difference in the growth of seeds in treated as compared with untreated fruits of the first cluster. Approximately 80 per cent of the treated ones contained fewer seeds than did the controls, and 50 per cent were entirely seedless. Even more striking results were observed when Marglobe plants were treated repeatedly with vaporous naphthoxyacetic acid. Ninety-eight per cent of the fruits on the first cluster were seedless; 92 and 80 per cent of those on the second and third clusters, respectively, were seedless; while only 6 per cent of those on the untreated fourth cluster were without seeds.

Although the treatment specifically inhibited the growth of seeds, it did not decrease the growth of locule contents other than the seeds, since the number of unfilled locules in either treated or untreated fruits was negligible (fig. 2). HOWLETT (5) reported that the use of indolebutyric acid also inhibited seed growth of greenhouse tomatoes and in some instances even increased the amount of gelatinous pulp.

Although treatment with naphthoxyacetic acid specifically inhibited growth and development of seeds of the Bonny Best variety, the vitamin C or ash contents of the fruits were not significantly altered as a result of treatment (table 1). There was no significant difference between the size, fresh weight, or percentage of solids in treated and control fruits, neither was there a noticeable difference in their color or flavor when ripe.

Summary

1. In experiments with tomato, the use of vaporized β-naphthoxyacetic acid under greenhouse conditions resulted in the development of a high percentage of seedless fruits (50–98 per cent). With the Marglobe variety the response was readily obtained by fumigating the plants in position when the earliest flowers of each successive cluster opened. This method, although causing temporary epinasty to a slight degree, did not appreciably affect vegetative growth of the plants. With Bonny Best variety slight increase in fruit set was observed, and this effect was limited to flowers of the cluster which were well developed but not yet open at the time of treatment.

2. With respect to vitamin C content, the nutritional value of ripe fruits that developed from treated flowers was equal to that of fully seeded untreated fruits grown under the same conditions.

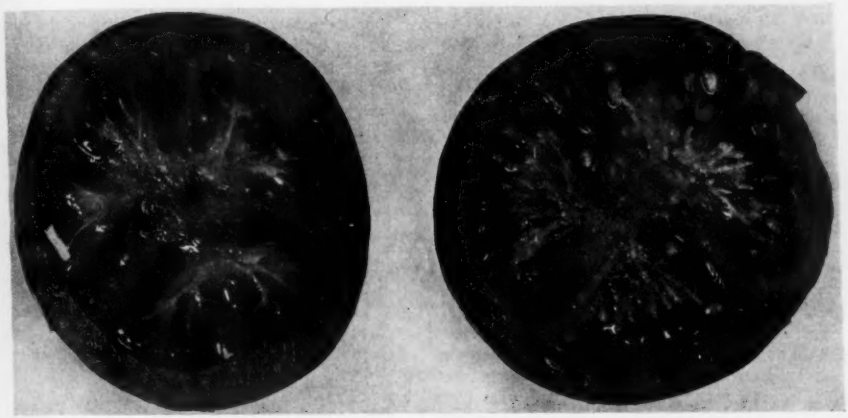

FIG. 2.—Ripe tomato fruits showing inhibited seed growth and amount of placental tissue and gelatinous pulp when previously treated with vaporized naphthoxyacetic acid (left) compared with untreated (right).

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CURRENT LITERATURE

The Reproductive Capacity of Plants. By E. J. SALISBURY. London: Bell and Sons, 1942. Pp. xi+244. Figs. 35. 30/.

The author presents data, many of them analyzed statistically, gathered during the past 15 years on the reproductive capacity of a species and the possible ecological importance of such capacity. The abundance of material adds greatly to the relatively small amount of information previously available. Among many others, the following points are made by the author:

1. The average and potential progenies of a species are as much a specific characteristic as are its average and potential heights, although, like the latter, subject to a considerable range of normal variation.

2. The seed or fruit weight tends to augment with the degree of shade to which the seedling is normally subjected. In general, it would appear that the more advanced the phase of succession with which the species is naturally associated the greater the amount of reserve food material provided in the seed.

3. The capacity to colonize in the face of competition appears to be associated with the amount of food reserve which the seed contains.

4. It would generally appear to be true that, for ecologically comparable species, the magnitude of the reproductive capacity is associated with the frequency and abundance, of which it is probably one of the determining factors. On the other hand, the magnitude of the reproductive capacity appears to bear little if any relation to the risks of mortality, although a certain minimum is obviously necessary to meet those risks, but probably, for most species at least, the seed output is considerably in excess of that minimum.

5. The lowest seed outputs characterize the herbaceous shade species of woodlands, in which the increased pressure of competition and shading to which the germinating seedlings are subject is met by increased seed weight and/or vegetative means of propagation.

6. For most species at least, the seed output is considerably in excess of that requisite for mere replacement of losses by death and sufficiently so to bear no obvious relation to the normal seedling mortality.

7. Some of the most successful species are those which exhibit a large seed output and possess also a means of vegetative propagation, whilst species in which vegetative propagation is the only or dominant method of reproduction are mostly associated with the more densely populated habitats and the later phases of the plant succession.

8. A high reproductive capacity, in the views hitherto orthodox, might be rightly held to be in the nature of a mark of failure, whereas the evidence furnished has shown that a high potential rate of increase is, indeed, rather a criterion of success.—E. J. KRAUS.

Plasmodiophorales. By JOHN S. KARLING. New York: Published by the author, 1942. Pp. ix+144. Illustrated.

The publication of a monograph, beautifully illustrated and comparative, of the difficult group of the Plasmodiophorales will be welcomed by botanists and plant pathologists. This group includes eight recognized genera: *Plasmodiophora*, *Tetramyxa*, *Sorosphaera*, *Sorodiscus*, *Spongospora*, *Ligniera*, and *Polymyxa*. Of these, *Plasmodiophora* is the best known.

The small size of the organisms, the unusual cytological phenomena, and the relations of the group to other fungi and of the members of the group to one another make them very confusing to the average biologist, and this effort to bring together, compare, and evaluate the widely scattered observations is to be commended. Even the terms used in describing them are so unusual that a glossary precedes the general discussion.

In the chapters on the general cytology of the group and of the various genera and species, the author is on familiar ground. The small size of the nuclei, cells, and spindle figures, together with the poorly defined outlines of the cells in host plants, make accurate observations difficult and lead to theorizing on life histories, alternation of generations, and to the inclusion of doubtful species in the various genera. The position of these excluded or doubtful species has not been indicated, however, and the reader wonders what the author would do with them.

Plant pathologists will find in the two chapters on clubroot of cabbages and other cruciferous plants, and that on powdery scab of potatoes, a more detailed account of the parasites and the diseases which they cause than may be found in the larger plant disease manuals. The chapter on clubroot discusses resistant and susceptible varieties and methods of control, with data and observations compiled from all previous investigators. The chapter on the phylogeny and relationships of the group and of its members is outstanding.

The plates and text figures are accurately and compactly redrawn from various publications, and

the diagrams illustrating the various theories on life histories are excellent. Bibliographies are supplied for each chapter and are as complete as existing world conditions make possible. The book will be indispensable to the cytologist, botanist, and plant pathologist.—B. F. LUTMAN.

Ornamental American Shrubs. By WILLIAM R. VAN DERSAL. New York: Oxford University Press, 1942. Pp. xiii+288. Illustrated. $4.00.

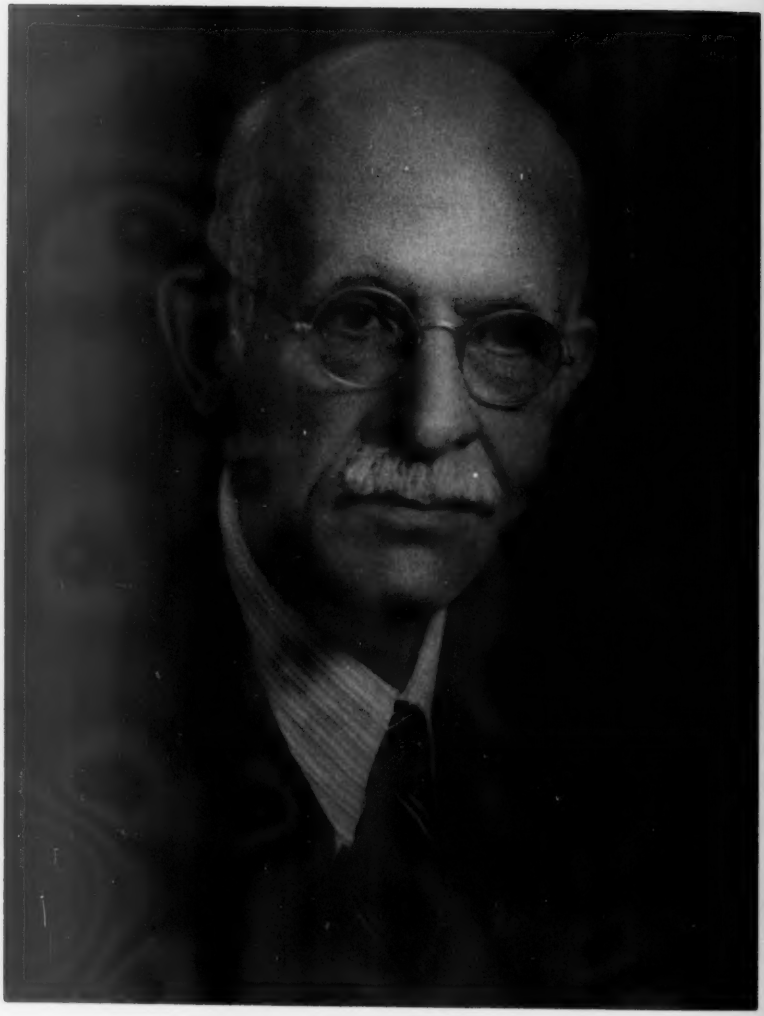
The book points out the possibilities for the enrichment and improvement of American gardens through the extended use of many more native shrubs than is now the case. The author briefly indicates what those who have tried to cultivate native shrubs quickly learn by experience—that many of them are both disappointing and difficult subjects under cultivation. Some of the most useful are given special mention. It is also undoubtedly true that additional varieties and forms may be selected which have greater horticultural value, including under that designation their relative freedom from insect and disease attack and their responses to horticultural practices such as cultivation, pruning, etc.

A valuable part of the discussion concerns the plant-growth regions of the United States; more than thirty are listed. Shrubs which are considered desirable horticulturally are presented in groups, based on their botanical classification, and are then described in greater detail. The suggestions as to the regions in which they are likely to succeed should prove particularly valuable.

The descriptions are in the main clear, objective, and relatively free from the poetic subjectivity and effusiveness which so frequently confuse discussions on ornamental plants. The illustrations are liberal in number, purposeful, and of outstanding merit and beauty. In places a somewhat flippant style adds nothing of value to the text. Some of the discussion dealing with plant exploration under the general heading of looting seems neither appropriate nor needful, if one considers the valuable information obtained as a result of transplantation of American flora abroad, and in turn the nearly incalculable gains from the introduction of many foreign species into America. The work is thoroughly readable, however, and makes a definite contribution to horticultural literature.—E. J. KRAUS.

Weed Control. By WILFRED W. ROBBINS, ALDEN S. CRAFTS, and RICHARD N. RAYNOR. New York: McGraw-Hill Book Co., 1942. Pp. xi+543. Illustrated.

This volume goes far toward filling a great need for information on weeds and their control. It is comprehensive in scope, both in type of subject matter treated and in the detail with which it is presented. Principles and methods of control, critical evaluation of some chemical treatments, and the handling of special problems are presented with sufficient consideration and detail to provide a clear and definite background for dealing with the weed problem. There is also a list of some weeds frequently encountered and regarded as of special economic importance. Data are presented by means of clear tables, graphs, and many excellent, fresh, meaningful illustrations. Running comments, backed by the experiences, contacts, and critical judgments of the authors combine to make a reference and guide of more than usual value.—E. J. KRAUS.

Forest Tree Seed. By HENRY IVES BALDWIN. Waltham, Massachusetts: Chronica Botanica Co., 1942. Pp. xvi+240. Figs. 28. $4.75.

The book deals comprehensively with the structure, development and origin, collection, cleaning and storage, germination, biotic enemies, and certification of tree seeds. There is also a chapter suggesting additional research problems. The material presented is critically examined, well documented, and there are fairly extensive lists of references to literature. Much of the discussion on harvesting, storage, and germination pertains with equal validity to many types of plants, so that the book has a wide field of interest and usefulness beyond the more immediate limits of species of trees.—E. J. KRAUS.